Metabolic Environment and Cellular Signaling in Haematopoietic Stem Cells and Progenitor Cells

Dissertation

zur Erlangung des akademischen Grades

Dr. rer. med.

an der Medizinischen Fakultät

der Universität Leipzig

eingereicht von:

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geb am 05.02.1989 in ABHA-Saudi Arabien

angefertigt an: Klinik und Poliklinik für Hämatologie und Zelltherapie,
Internistische Onkologie und Hämostaseologie Leipzig
in Kooperation mit: Institut für Klinische Immunologie Leipzig

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Beschluss über die Verleihung des Doktorgrades vom: February 25, 2020

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Decision on the awarding of the doctoral degree from: February 25, 2020

Abstract

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The University of Leipzig, Dissertation

58 P., 77 Ref., 59 Abb., 3 Appendix

Haematopoietic bone marrow is functionally divided into areas supporting the maintenance and self-renewal of stem cells on the one hand and the proliferative differentiation of progenitor cells on the other. It is proposed that this functional organization is determined not just by the signaling environment provided by the local presentation of growth factors, but also by metabolic variables such as glucose, nicotinamide (NAM) and osmolarity, which may vary with the position relative to arterioles and sinusoids. A key issue here is whether or not variations in the metabolic environment of a progenitor cell alter the response of that cell to growth factors.

Firstly, it was found that the simple addition of NAM in the absence of added myeloid growth factors is sufficient to support differentiation of multipotent progenitors to granulocyte-like cells, demonstrating that the nutrient composition of medium can in principle influence the balance between self-renewal and differentiation.

Secondly, a relatively small increase in the salt concentration in the growth medium increased both the proportion of early progenitors and the level of JAK2-STAT5 signaling via the IL-3 receptor, which is associated with self-renewal rather than differentiation. This shows that physico chemical conditions can also influence the differentiation of haematopoietic progenitor cells.

Thirdly, decreasing the glucose concentration over the range 5 – 0.1mM progressively decreased the pERK1/2 response to IL-3, while pSTAT5 levels actually increased under low glucose. An investigation of the mechanism by which JAK-STAT activity is maintained under low energy conditions revealed a requirement for Nme2, a nucleotide diphosphate kinase that is differentially expressed in progenitor cells and that has been implicated in myeloid leukemia. Based on these results, it is proposed that a specific association between Nme2 and the JAK2 STAT5 pathway enables local regeneration of ATP and maintenance of signaling.

This work demonstrates how the nutrient and physico-chemical environment can indeed affect the self-renewal versus differentiation of haematopoietic progenitor cells in vitro, consistent with the proposed existence of metabolic niches in vivo that contribute to the organization and control of haematopoiesis. The identification of Nme2 as a key link between metabolic and signaling activities should enable more detailed analysis of these relationships in the future.

Dedication

This work is dedicated to

The soul of my father

To my mother

To my sister and brothers

To my wife and my little daughter

To my family and my friends who supported me

To all patients suffering from cancer and haematological disorders

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List of Abbreviations

°C	Celsius
ADP	Adenosine diphosphate
AKT	protein kinase B
AMPK	AMP-responsive kinase
c-Jun	a protein that in humans is encoded by the JUN gene
CM	conditioned medium
cMyc	Cellular virus, Myelocytomatosis
DNA	Deoxyribonucleic acid
dsNme2	Double strand Non-metastatic 2
EPO	Erythropoietin
ERK	Extracellular Signal Regulated Kinase
Ets-1	a protein that in humans is encoded by the ETS1 gene.
FDCP-Mix	Factor Dependent Cell Paterson
FLT3	FMS like tyrosine kinase 3
GCSF	Granulocyte Colony Stimulating Factor
GCSFR	Granulocyte Colony Stimulating Factor Receptor
GFP	Green Fluorescent Protein
Grb	Growth Factor Receptor Bound Protein
GTP	Guanosine Triphosphate
HIF-1α	Hypoxia Inducible Factor
HSCs	Hematopoietic Stem Cells
IL	Interleukin
JAK	Janus Associated kinases
JNK	c-Jun N-terminal kinases
MAPK	Mitogen Activated Protein Kinases
M-CSF	Macrophage Colony-Stimulating Factor
MEK	Mitogen Activated Protein Kinases
mOsm/Kg	milliosmoles per kilogram
MPPs	Multipotent Progenitor cellS
mRNA	Messenger Ribonucleic Acid
mTOR	mammalian Target Of Rapamycin
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide- Reduced form
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAM	Nicotinamide
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B-cells
Nme2	Non-metastatic 2 /Nucleoside diphosphate kinase B
PBS	Phosphate-Buffered Saline
pGhU6	pLeGO-hU6 Lentiviral Gene-Ontology with humanized U6 promotor vector
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3-kinase
qRT-PCR	quantitative Real-time Polymerase Chain Reaction
RAF	Rapidly Accelerated Fibrosarcoma

RAS	Rat sarcoma
ROS	Reactive Oxygen Species
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SCF	Stem Cell Factor
shNme2	A short hairpin RNA or small hairpin of Nme2 mRNA
shRNA	A short hairpin RNA or small hairpin RNA
siRNA	Small interfering RNA
SOS	The Drosophila Protein Son Of Sevenless
SREBP	Regulatory Element Binding Protein
STAT	Signal Transducer and Activator of Transcription
TPO	Thrombopoietin
TF	Transcription Factors
UCB	Umbilical cord Blood
Wnt3a	Wingless-Type MMTV Integration Site Family, Member 3A

1. Introduction and literature review

1.1 Haematopoietic stem cells and progenitor cells

The adult human blood system is maintained by replacement of more than 10^{11} cells per day through continuous haematopoiesis: A hierarchical differentiation and proliferation system in the bone marrow. At the top of the hierarchy are haematopoietic stem cells (HSCs) that cycle slowly to generate on average one replacement HSC and one multipotent progenitor. The multipotent progenitors then proliferate more rapidly, generating daughter cells that follow programs of commitment to the lymphoid, myeloid or erythroid lineages. Under some conditions (during development or after damage), the pool of HSCs can be increased by symmetric divisions in which one stem cell produces two (Nakamura-Ishizu et al. 2014; Ng and Alexander 2017; Pinho and Frenette 2019).

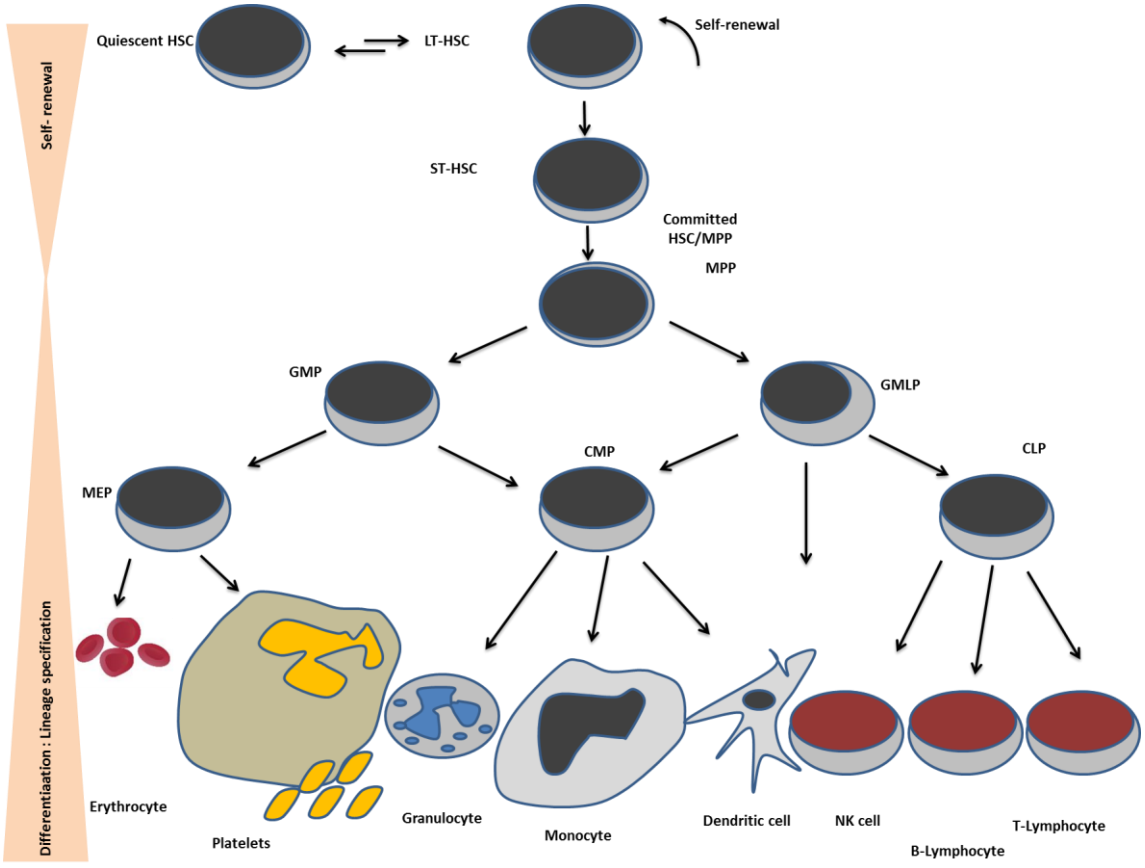


Fig 1-1 Summary of haematopoietic hierarchy and stem cell fate.

It is important to understand the ways in which this process is regulated to maintain a balanced output throughout life, even when the demand for particular cell types changes due, for example, to infection, injury or simply change of altitude. A detailed understanding of these control mechanisms will be required for the efficient recognition and treatment of haematological disease. Most attractive would be the possibility of amplifying HSCs ex vivo, since the number of HSCs is a limiting factor in some transplantation strategies (e.g. using umbilical cord blood stem cells for adults, or repeating transplantations from the same

donor). Also, the quiescent or slow-cycling status of the earliest stem cells limits the efficiency of viral gene therapy. The efficient generation of erythrocytes and granulocytes in vitro is another important goal, as this could offer a way of producing well-characterized, safe cells for therapeutic applications by “cell-pharming” in bioreactors rather than relying on blood donations. Finally, a characterization of the mechanisms controlling normal haematopoiesis promises to reveal valuable information about the differences between normal cells and their leukemic counterparts, properties specific to leukemic cells being potential targets for new and selective diagnostic/therapeutic approaches.

With these aims, there has been much interest over the years in characterizing the mechanisms of haematopoiesis. Most of the effort has been spent on isolating and characterizing a wide range of growth factors that have specific effects on stem/progenitor or differentiating cells. Some of these factors, most notably erythropoietin (EPO), thrombopoietin (TPO) and granulocyte colony stimulating factor (G-CSF) are in widespread use to boost the numbers of erythrocytes, platelets or granulocytes in patients, although use of the same factors in bioreactors to drive production of therapeutic cells in vitro has proven very challenging. Other factors (including stem cell factor (SCF), Flt-3-Ligand, Wnt3a, Hedgehog and a range of interleukins) clearly have effects on stem/progenitor cell compartment but have not been translated into widespread clinical benefit (Lento et al. 2013; Lampreia et al. 2017; Xu et al. 2018). One of the reasons for the limited impact of growth factors in vitro is that their action may well rely on having an appropriate metabolic environment (Cross et al. 2008). Both supportive growth factors and the appropriate metabolic environment are supplied locally by stromal cells in the bone marrow (Kohli and Passegué 2014). The fate of a stem/progenitor cell is then most likely decided both by the growth factors that are present and the metabolic conditions in which that signal is seen. In order to understand the control of haematopoiesis, it is therefore likely to be informative to focus on the link between metabolism and signaling in stem and progenitor cells.

1.2 Role of metabolism in normal and leukaemic HSC and progenitor cells

The stroma that supports the various stages of blood cell production is formed by a number of interacting cell types including mesenchymal stromal cells, osteoblasts, adipocytes, neural cells, and endothelial cells of the arterioles and sinusoids (Kumar et al. 2018). Given the high level of biosynthetic metabolism occurring in the space between arterioles, sinusoids and bone surfaces, regional variations can be expected in the concentration of the substrates and products of metabolism such as carbohydrates, amino acids, fatty acids oxygen and carbon dioxide. This means that a haematopoietic cell is likely to be exposed to different cell-cell and cell-matrix contacts, growth factor signals and metabolites depending on its position in the marrow architecture. This raises the question of whether metabolic compartments within the marrow help to localize organize and regulate haematopoiesis.

There are a number of indications that this is indeed the case. Firstly, although the consistency of haematopoietic marrow has made it difficult to define the complex architecture in detail, it is indeed clear that distinct stages and lineages are located to

distinct regions. For instance, HSCs tend to be located near to vessel walls or endosteal surfaces (Morrison and Scadden 2014),while erythropoiesis and megakaryopoiesis are associated with sinusoids and the production of granulocytes is associated with arterioles (Naito et al. 1992).

Secondly, studies of haematopoietic cell metabolism reveal shifts in the pattern of activity and preferred substrates as stem cells progress through commitment and differentiation (Kohli and Passegué 2014; Agathocleous and Harris 2013). The current consensus is that quiescent stem cells rely largely on glycolysis to provide the energy that they require and that glycolytic metabolism is supported by the hypoxic environment of the stem cell niche. The hypoxia inducible factor HIF-1α is involved in the induction of genes required for glycolysis as well as the suppression of mitochondrial metabolism, hence limiting the production of reactive oxygen species (ROS) as a potentially damaging by product of oxidative phosphorylation (Simsek et al. 2010). In this way, the stem cell pool is protected from accumulating long term damage. As the cells become progressively more activated, mitochondrial metabolism is increased in order to provide the biosynthetic metabolism required for proliferation. In the early stages of activation, fatty acid oxidation (by which the mitochondria generate energy but no biosynthetic intermediates) has been shown to be necessary in order to prevent commitment and to maintain the stem cell phenotype in at least one of the daughter cells (Ito et al. 2012). Subsequent lineage commitment and differentiation involve the high level activation of mitochondrial metabolism with the associated generation of ROS (Fig1-2) and differential use of amino acid and carbohydrate substrates, glutamine being particularly important for erythropoiesis, while glucose appears to be the preferred substrate for myelopoiesis (Oburoglu et al. 2014; Billing et al. 2017).

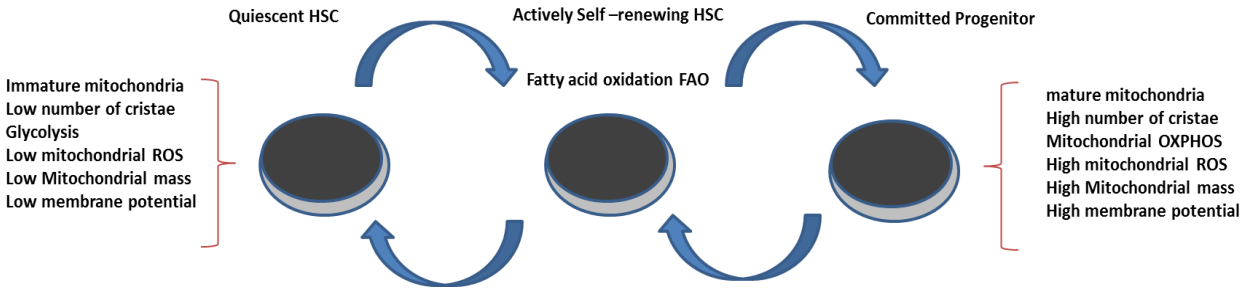


Fig 1-2 Changes in metabolism during stem cell commitment.

The fact that haematopoietic progenitors change both their location and their metabolic activity as they undergo commitment and differentiation is consistent with the idea that their fate may be influenced by their metabolic environment, which is defined not only by the local concentration of nutrients and waste products but also by physico-chemical parameters such as oxygen concentration, pH and osmolarity. Each of these is likely to vary with position in the marrow. While the effects of osmolarity on haematopoiesis have not been studied in any detail, it should be noted that the optimal osmolarity for the culture of haematopoietic cell lines (which at 300 to 320 mOsm/kg corresponds roughly to that of plasma) is not optimal for the maintenance of stem cell activity in long term bone marrow

cultures. Here, a higher osmolarity of 350 mOsm/kg supports more robust activity and more stable cultures (Coutinho et al 1993).

The proposed influence of cell metabolism on stem and progenitor cell fate raises the question of whether it is possible to influence fate using interventions that change the metabolic environment. In support of this, there have been a number of reports that the addition of nicotinamide (NAM, vitamin B3) selectively affects the fate of haematopoietic cells both in vitro and in vivo. NAM is the precursor of nicotinamide adenine dinucleotide (NAD+), a coenzyme with a wide variety of activities in both signaling and metabolism. NAM supplementation shifts the balance of macrophage differentiation (Weiss et al. 2015) and increases the granulocytic differentiation of the human HL60 myeloid leukemia cell line and of primary CD34+ stem/progenitor cells(Skokowa et al. 2009). This was found to involve the NAD+-mediated activation of sirtuin-1 and induction of the GCSF and GCSF-R genes, leading to an autocrine/paracrine support of granulopoiesis. NAM supplements given to healthy human volunteers also increased granulocyte output, so NAM has a pro-granulopoietic effect also under physiological conditions. NAM has also been reported to selectively preserve CD34+ cells and stem cell activity in umbilical cord blood mononuclear cells (Peled et al. 2012). The promotion of sirtuin (NAD+ dependent histone deacetylase) activity has been implicated in this effect, but the details of the mechanism and the relationship to the granulocyte differentiation effect remain unclear. However, the fact that NAM-derived NAD+ is also an essential cofactor in a wide range of metabolic processes and a substrate for signaling intermediates. (Fig 1-3) suggest that the effects on haematopoiesis may not be solely down to the activation of sirtuins, but could involve a combination of effects at the levels of metabolism, signaling and gene expression. The NAM response of haematopoietic progenitor cells therefore provides an opportunity to investigate more closely links between signaling and metabolism in the control of cell fate.

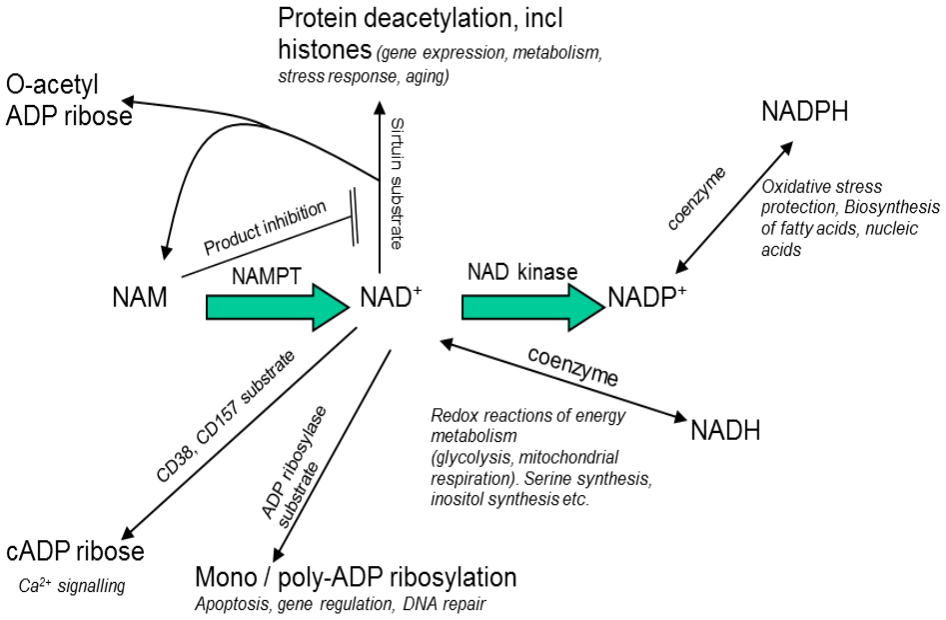


Fig 1-3: NAM-derived NAD+ is involved in a variety of metabolic and signaling pathways as well as in the control of gene expression.

The emerging role of metabolism as a determining influence on haematopoiesis raises the question of whether there are differences between normal and leukemic cells in this respect that may be used to target the treatment of leukemias. In general, leukemic cell populations tend to have either high glycolytic activity, in common with many solid tumors (Chen et al. 2014), or high levels of fatty acid oxidation (Moreno 2015; Jones et al. 2018). However, although these activities may provide a growth and survival advantage to the bulk of the leukemic cell population, it is the small proportion of leukemic stem cells that are most relevant to long term prognosis and here it is still not clear to what extent the self-renewal of leukemic stem cells may differ from that of normal stem cells in terms of metabolic activity and requirements (Ito and Suda 2014; Stuani et al. 2019).

1.3 Interaction between Signaling and Metabolism

Although metabolic and signaling pathways have been analyzed largely independently in the past, there are three levels at which they may interact to coordinate cell responses.

Firstly, metabolic enzymes can be activated or repressed in response to receptor-mediated signaling. Foremost among the signaling pathways linking to metabolism in this way is the PI-3K-/Akt/mToR pathway which is activated by receptor tyrosine kinases and induces the expression of genes for glucose uptake and glycolysis (via activation on the hypoxia-inducible factor HIF-1α) and genes for the pentose phosphate pathway and lipid synthesis via the activation of sterol regulatory element-binding protein SREBP, (Yecies and Manning 2011). Secondly, these signaling responses can be modulated by nutrient sensors that take account of the availability of the metabolites and energy. For instance, the enzyme AMP-responsive kinase (AMPK) responds to the increase in AMP levels at low energy levels by inactivating enzymes required for biosynthetic pathways with high energy demand, but also targets transcription factors involved in signaling response (Mihaylova and Shaw 2011). Finally, since a number of key metabolites serve as substrates for regulatory modifications (phosphorylation, methylation, acetylation, glycosylation or ADP-ribosylation), it is feasible that these modifications themselves may be substrate-limited, thus linking signaling responses directly to the metabolic environment. In support of this principle, there is strong evidence that high levels of α-ketoglutarate contribute to the demethylating activity involved in maintaining potential in cultured pluripotent stem cells (Carey et al. 2014) . Here, it should be borne in mind that the culture of pluripotent stem cells in nutrient-rich media is non-physiological and pluripotency is a very short lived state in the physiological environment inside a pre-implantation blastocyst. In contrast, adult HSCs maintain their pluripotency in vivo over very long periods of low activity. In this respect, it is noteworthy that many of the cell-cell signals that are associated with the maintenance and amplification of HSCs during development or in the niche tend to trigger short and direct transduction pathways with few intermediates and a low energy requirement. The Notch Receptor, for instance, need only undergo proteolytic cleavage in order to create a transcription factor, while canonical Wnt signaling achieves a transcriptional response by reducing rather than increasing target (ß-catenin) phosphorylation. Once the cells leave the niche and are exposed to soluble ligands, it is possible that the activity of specific signaling pathways will

depend on the level of energy available and that the metabolic environment may in this way influence the balance between self-renewal and differentiation. Two signaling pathways of particular interest in this respect are the JAK-STAT and Ras-MAPK cascades. These pathways are commonly triggered by the same ligand-bound receptors but are associated with different effects.

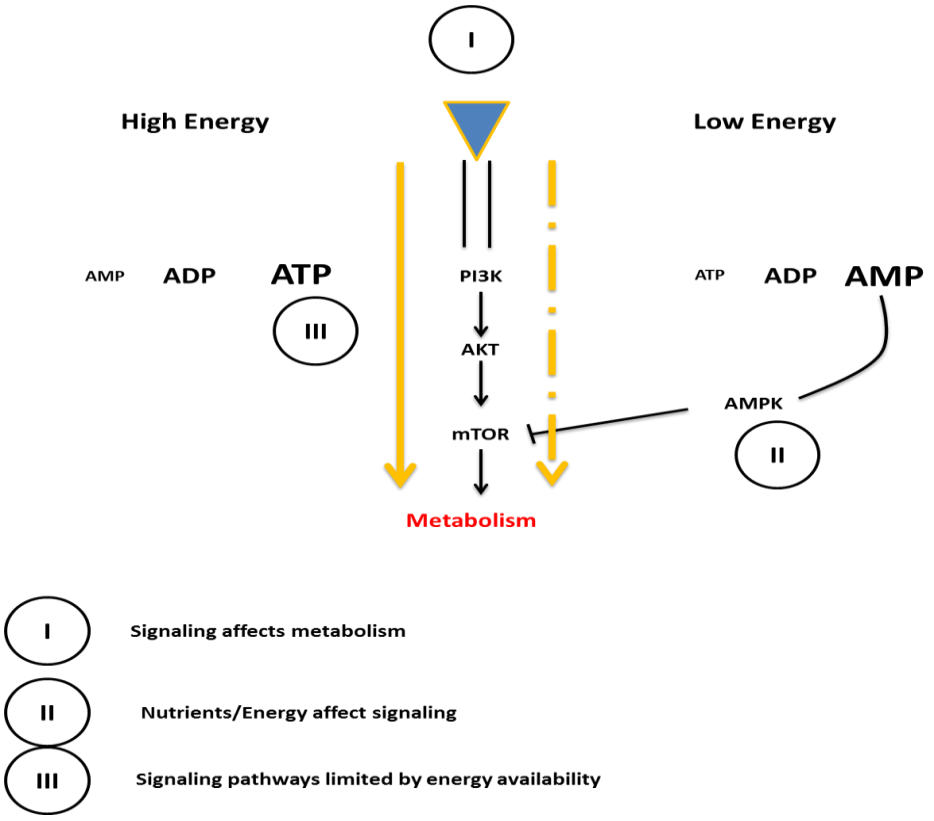


Fig 1-4 Potential connections between signaling and metabolism.

1.4 Features of JAK-STAT5 and Ras-MAPK signaling

The Janus kinase and Signal Transducer and Activator of Transcription 5 pathway (JAK-STAT5) is the main pathway for self-renewal in normal and leukaemic HSC is comparatively short, with the JAK-mediated phosphorylation of STAT5 converting it directly to a transcription factor (Aaronson and Horvath 2002; Schepers et al. 2012; Han et al. 2009). In contrast, the Ras-MAPK pathway (also known as the Ras-Raf-MEK-ERK pathway) which is ubiquitously involved in the regulation of cell proliferation, differentiation and death (Hsu et al. 2007; Matsuzaki et al. 2000; Miranda et al. 2005; Pearson et al. 2009; Richardson et al. 2015; Woessmann et al. 2004) involves a multifactorial kinase cascade and a correspondingly high energy investment.

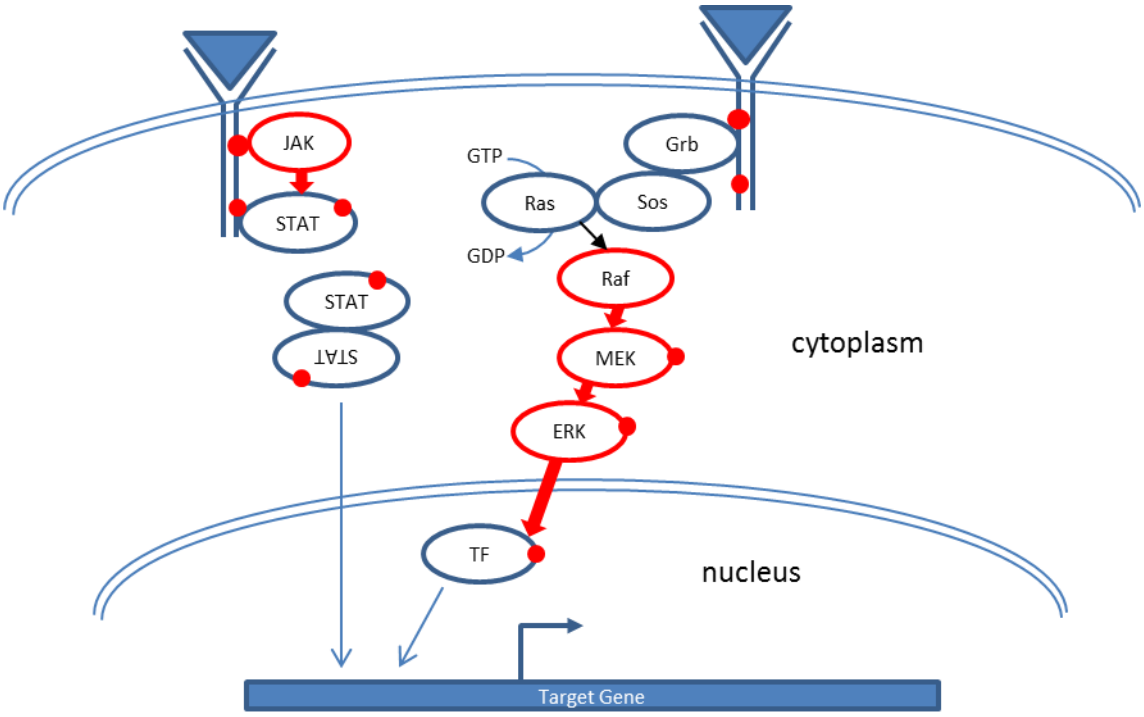


Fig 1-5 The JAK-STAT and Ras-MAPK pathways.

1.4.1 JAK-STAT5 pathway

STAT5 is broadly expressed throughout the normal and leukaemic haematopoietic system (Han et al. 2009), where it can be activated by diverse cytokine and growth factor receptors including those of the interleukin 3 (IL-3) family, comprising IL-3 itself, IL-5 and granulocyte macrophage colony stimulating factor GM-CSF; the common gamma-chain receptor family (IL-2, IL-7, IL-9, IL-12, IL-15); as well as a wide range of single chain receptors for EPO, TPO, G-CSF, macrophage colony stimulating factor (M-CSF), SCF and many others (Ghanem et al. 2017; Han et al. 2009; Wierenga et al. 2006). In the vast majority of these cases, including that of the IL-3 receptor family, STAT5 activation requires the activation of a JAK kinase, which is recruited to the ligand-bound receptor following ligand binding and receptor autophosphorylation. JAK can then phosphorylate both the receptor at a separate site (to recruit STAT) and then STAT itself. Since phosphorylated STAT then merely dimerises to form a transcription factor, the energy input into the JAK-STAT pathway is relatively low (Fig 1-5).

1.4.2 RAS-MAPK-ERK pathway

The RAS-RAF-MEK-ERK pathway is a ubiquitous, multi-step pathway that is triggered by ligand binding to tyrosine kinase receptors and is involved in the regulation of cell proliferation, differentiation and death. The major components of the pathway include a small G protein (RAS) and a series of three protein kinases (RAF, MEK, ERK). Following ligand binding, dimerization and autophosphorylation, the phosphorylated receptor recruits an adaptor protein (Grb) which in turn interacts with the GTPase-activating protein (SoS). It is the SoS protein that is responsible for activating Ras by charging it with GTP. Activated Ras in turn activates Raf, which is the first in a cascade of kinases. The final cytoplasmic kinase reaction targets and activates a Map Kinase (MAPK). There are three well-described

subfamilies of MAPKs: the extracellular flag managed kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases (Cargnello and Roux 2011). The activated MAPK translocates to the nucleus where it activates transcription factors that regulate gene expression (McCain 2013). In the case of ERK1/2, the final targets in the nucleus include a range of transcription factors, such as Ets-1, c-Jun, c-Myc and NF-κB (Nakano et al. 1998). As summarized in (Fig 1-5), the Ras-MAPK pathway requires a complete kinase cascade which amplifies the signal at the cost of a high energy demand, while the JAK-STAT pathway involves just a single soluble kinase to activate transcription. Given that JAK-STAT5 activity In haematopoietic progenitor cells is associated with self-renewal (maintenance of the stem cell phenotype in at least one daughter cell) while Ras-MAPK signaling is more closely associated with proliferative differentiation. It is feasible that the balance of activity between these two pathways influences cell fate in response to available energy levels.

1.5 The FDCP-Mix cell culture model

The association of metabolism and signaling can be approached most directly in a haematopoietic progenitor cell culture system in which both the JAK-STAT and Ras-MAPK pathways can be activated by the same growth factor receptor stimulation. The murine Factor Dependent Cell Paterson Mixed Potential (FDCP-Mix) system offers precisely such a system. FDCP-Mix cells are karyotypically normal, non-leukaemic, multipotential progenitor cells that undergo differentiation into all myelo-erythroid lineages in the presence of suitable stroma or lineage-specific growth factors (Heyworth et al. 1995; Spooncer et al. 1986). In the presence of high concentrations of interleukin 3 (IL-3), differentiation is blocked and the cells amplify as a population of self-renewing multipotent progenitors, although even in this state FDCP-Mix populations are heterogeneous, with 2-5% of cells being capable of initiating colonies in semi-solid medium. The continued culturing of the FDCP-Mix cells for more than 2 months can result in the loss of erythroid differentiation potential that is often followed by the acquisition of a pre-leukaemic transformed phenotype, increased clonogenicity and a complete differentiation block. For these reasons, it is routine procedure to work with FDCP-Mix cells within 2 months of thawing (Just et al. 1993; Spooncer et al. 1986). The near-physiological properties of the FDCP-Mix cells during the early stages of culture make them a powerful model system in which to study the mechanisms of self-renewal and differentiation (Fairbairn et al. 1993; Pierce et al. 2002; Göttig et al. 2006; Billing et al. 2017).

1.6 Hypothesis and Aims

Based on the reasoning outlined above, it was hypothesized that the control of self-renewal, commitment and differentiation of haematopoietic progenitor cells in response to haematopoietic growth factors can be influenced by the metabolic environment, which varies depending on the position in the bone marrow. In this way, haematopoiesis may be organized into compartments defined by the availability of substrates for catabolic (energy producing) and anabolic (biosynthetic) metabolism and cofactors, and by physicochemical parameters such as oxygen tension, pH, and osmolarity. Specifically, it was hypothesized that there is a bias towards JAK-STAT (self-renewal) signaling in low energy environments.

The aims of this work were to test this hypothesis in the following ways:

- To determine the effects of metabolic restriction and inhibition on the balance between JAK-STAT and Ras-MAPK signaling in multipotent progenitor FDCP-Mix cells responding to IL-3.
- To examine the dependence of signaling on changes in osmolarity
- To determine the effects of nicotinamide on the self-renewal and differentiation of haematopoietic progenitor cells

2. Material and Methods

2.1Cell culture

The FDCP-Mix Cell line (Spooncer et al. 1986) was cultured in a maintenance / self-renewal medium consisting of Iscove's Modified Dulbecco's Media (IMDM, PAN Biotech GmbH, Germany, cat. no. L-06-054-212-001, glucose and glutamine-free supplemented with 10% IMDM medium conditioned by the IL-3 producing murine myeloma cell X63AG8.653 (Karasuyama and Melchers 1988), 20% horse serum (Gibco lot no. 818358), 5mM glucose (Sigma Aldrich, Germany cat no G7021-100G) and 2mM glutamine (Sigma Aldrich cat no G8540-25G). In some cases, 10U/ml recombinant mIL-3 (PEPROTECH cat no 210-33, USA) as used instead of IL-3 conditioned medium (cm). The osmolarity of the growth medium was measured using a cryo-osmometer (Gonotech, Germany) and adjusted to 320mOsm/kg unless otherwise stated. Cells were seeded at 6 x 10^4 per ml and passaged every 2 days to keep the cell density between 6 x 10^4 and 4 x 10^5 /ml. Depending on the requirements of the experiment, media were made with varying glucose concentrations (0mM to 10mM) and osmolarities (320, 340 and 360mOsm/kg) and were supplemented with metabolic inhibitors 2 deoxyglucose (2DG, Sigma –Aldrich cat no D6134-1G) and/or rotenone (rot, Sigma-Aldrich R8875-1G), or nicotinamide (NAM, Sigma NO636-100G, USA) as described in the results sections.

To differentiate the FDCP-Mix cells along myeloid lineages, self-renewing cells were harvested, washed twice in phosphate-buffered saline (PBS) to reduce the concentration of IL-3, then reseeded at 6 x 10^4 /ml into IMDM supplemented with 10% fetal bovine serum (FBS) plus 1u/ml recombinant murine IL-3 (PEPROTECH cat no 210-33, USA), 1000U/ml G-CSF (PEPROTECH cat no 210-23) and 50U/ml GM-CSF (PEPROTECH cat no 315-03). For erythroid differentiation, washed cells were seeded at 6 x 10^4/ml into IMDM supplemented with 10% FCS, 2U/ml mIL-3 (PEPROTECH cat no 210-33, USA), 3U/ml erythropoietin (EPO, PEPROTECH cat no 100-64, USA), and 0.2mM Hemin (Sigma-Aldrich H9039). Differentiation was assessed after 7 days of culture by FACS analysis of surface markers (see below) and by morphological examination of cytospun cells.

2.2 Cytospins

Cells (6x10^4/ml) were prepared for cytological examination by cyto-centrifugation onto pre-labelled glass slides using a Hettich - Universal 16 cytospin. The dried and fixed cells were stained for 10 minutes in the dark in solution A for erythroid progenitors (100 ml) : 50 ml O-Dianisidin 0.2 g /100 ml Methanol (Sigma Aldrich cat no . D9143) 10 ml Nitro ferri cyanide $Na_2Fe(CN)_5$ $NO.H_2O$ 1g/100 ml deionized water (Sigma Aldrich cat no. 228710) , 10 ml 30% H_2O_2 and 10 ml H_20. Then, the slides were washed in running deionized water and transferred to solution B (May-Grünwald stain ROTH Art no . T863.2) for 5 minutes followed by a repeat wash in deionized water. Lastly the slides were stained with solution C (1:20 Giemsa stain ROTH Art no . T862.1). Microscopical examination was done using Zeiss Vert.A1 and photographs was taken using AxioCam ICc1 camera.

2.3 Colony-forming cell Assays

FDCP-Mix cells ($3.3x10^3$) in 10ml self-renewal complete media IMDM were mixed with 1ml melted 3.3% agar (BactoTMAgar Ref no.214010,France) to final concentration of 0.33% agar. The cells were then plated out of this mixture in to 3 well plate (3 mls per well). The plates were incubated in 5% CO_2 in air at 37 °C for 7 days .The colony assessment was done at 20X magnification using a Zeiss Vert.A1 microscope. The colony was recognized as a group of > 50 cells as is described by (Heyworth and Spooncer 1993).

2.4Cryopreservation and thawing of cells

FDCP-Mix cells that had been in culture for 2 days were spun down at 800xg for 10 minutes. Then, the cells were re-suspended gently to a final density of 2 x 10^6/ml in 90% HS and 10% dimethyl sulfoxide DMSO and transferred in 1ml aliquots to labelled cryopreservation vials. The vials were transferred to a "Mr Frosty" freezing container and placed at -80 °C overnight for slow freezing. On the following day, cells were transferred to the gas phase of liquid nitrogen for long term storage.

For the thawing process, frozen vials were placed in a 37 °C water bath and agitated gently. As soon as the mixture was thawed, it was transferred immediately to a 50ml falcon tube. Ten ml of complete media was gently added drop wise over a period of 10 minutes to keep the osmotic gradient between the cell interior and exterior as constant as possible. Cells were left at room temperature for 10 minutes to recover. In the next step, the cells were spun down at 800xg for 10 minutes, the supernatant was removed and the cells were re-suspended in 10 mls of maintenance medium and incubated overnight at 37 °C, 5% CO_2. The next day, the dead cells were removed by density gradient centrifugation over Pancoll (PAN cat no P-04-60500, Germany) according to the manufacturer's instructions. Finally the recovered cells were re-suspended at $6x10^4$ /ml in maintenance medium and returned to the incubator. FDCP-Mix cultures were passaged every two days.

2.5 Intracellular Phosflow analyses for pSTAT5 and pERK1/2

To measure the pSTAT5 and pERK1/2 responses to IL-3 stimulation, FDCP-Mix cells were first washed with 1xPBS and incubated for 2 hours in medium without IL-3. At the end of this period, 100U/ml mIL-3 was added to all cultures except for the unstimulated control. After 5-30 minutes (depending on the experiment), 5 X 10^5 cells were fixed with Lyse/Fix buffer (BD Phosflow™ Lyse/Fix Buffer 5X cat no. 558049, SanDiego,CA) for 10 minutes at 37°C and then immediately centrifuged at 800xg and washed with 4 ml 1XPBS. Cells were stained with Fixable Viability Stain 780 (BD Horizon™ cat no. 565388) according to the manufacturer's instructions. Cells were washed 2 times with staining buffer containing 0.1%NaN_3 (Applichem cat no.22628-22-8, Germany). Permeabilization was carried out with 1 mL BD Phosflow™ Perm Buffer III cat no.558050) on ice for 30 min. After washing with staining buffer, cells were stained with BD Phosflow™ Alexa Fluor® 647 Mouse Anti-Stat5 (pY694) clone 47 (BD Biosciences cat no. 612599) and BD Phosflow™ Alexa Fluor® 488 Mouse Anti-ERK1/2 (pT202/pY204) clone 20A (BD Biosciences cat no. 612592). Isotypes and negative

(Unstimulated W/O IL3) controls were used to set the gates. The isotype controls used were (Alexa Fluor 647 Mouse IgG1, κ (BD Biosciences cat no. 566011) and Alexa Fluor® 488 Mouse IgG1 κ (BD Biosciences cat no. 557721). Flow cytometry was performed on a FACS Verse (BD).

2.6 Isolation and storage of human CD34$^+$ stem and progenitor cells

Umbilical cord blood donated for research purposes was obtained from Vita 34 AG, following informed consent of the parents. The blood was first transferred to a T75 culture flask, then diluted x2 with PBS. For each 40ml of diluted blood, a 50ml Falcon was prepared with 12ml Ficoll and up to 40ml of cell suspension layered carefully over the ficoll. Density gradient centrifugation was carried out for 35min at RT and 1000g without brake. The gradients were transferred carefully to the laminar flow box and each interphase of MNC was transferred by using a Pasteur pipette into one fresh 50ml Falcon. PBS was then added to each Falcon tube to 50ml to wash the remaining ficoll off the cells, and the cells recovered by centrifugation at 1000g 10min RT (brake on). The supernatant was removed using a 25ml pipette, then each pellet re-suspended in 5ml PBS before pooling maximum 5 x 5ml into a single Falcon and repeating the PBS washing step. Each pellet was then re-suspended in 10ml PBS, and all suspensions pooled to a single Falcon. A small volume of suspension was removed for cell counting using a hemocytometer and the remainder centrifuged once again at 1000g for 10 min at room temperature. The final pellet was re-suspended at a concentration of 10^7 cells per ml in PBS + 10% BSA on ice. When the cells were completely re-suspended, an equal volume of PBS + 10% BSA + 10% DMSO was prepared and added slowly, to give a final suspension of 5 x 10^6 cells/ml in PBS + 10% BSA + 5% DMSO on ice. The cells were then distributed to pre-labelled cryovials in 1 ml aliquots and placed directly into a Mr. Frosty (Thermo-Fisher, Germany) freezing container at 4^{o}C. The Mr. Frosty was then placed in a -80^{o}C Freezer overnight before transferring the frozen cells to the gas phase of liquid nitrogen. Upon thawing (described above) , the MNCs were cultured in serum free IMDM media with 50ng/ml SCF (PEPROTECH, cat no 300-07, USA), 50ng/ml TPO (PEPROTECH, cat no 300-18, USA) and 50ng/ml FLT-3(PEPROTECH, cat no 300-19,USA).The cells were incubated in 5% CO_2 and 18%O_2 at 37 °C overnight to recover. In some cases, CD34+ve cells were purified using indirect CD34 Microbead kit (Milteny Biotec , order no. 130-046-701, Germany). First, the MNCs cell number were determined then, following a centrifugation step at 300xg for 10 minutes, the cell pellets were re-suspended in 400µl staining buffer. After that, 100µl FcR blocking reagent was added then the CD34+ cells were indirectly magnetically labeled using 100µl hapten-conjugated primary monoclonal CD34 antibody. The cells were incubated for 15 mintues at 4°C. After a further centrifugation step at 300xg for 10 minutes, the cells were re-suspended in 400µl buffer and 100µl of anti-hapten MicroBeads were added and the mixture was incubated for 15 minute at 4 °C. Following another centrifugation step as described above, the cells were re-suspended in 500µl of the staining buffer. The cell suspension was loaded onto a MACS column which was placed in the magnetic field of a MACS separator. The magnetically labeled CD34+ cells were retained within the column. The unlabeled cells were run through; the cell fraction was thus depleted

of CD34+ cells. After removing the column from the magnetic field, the magnetically retained CD34+ cells were eluted as the positively selected cell fraction. the experiments were performed and analyzed as is described in the results section 3.4

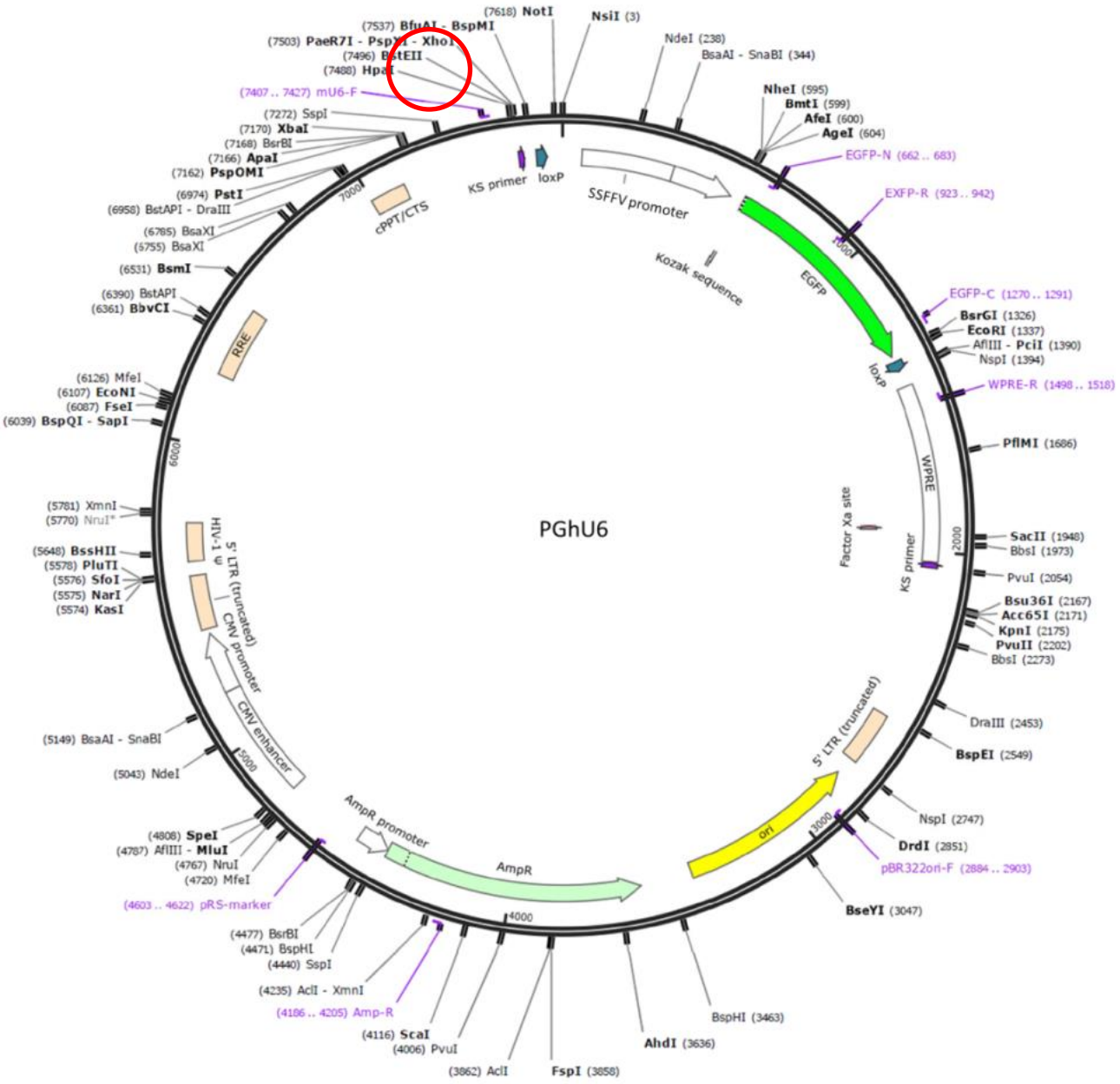
2.7 Lentiviral backbone plasmid cloning, amplification, isolation and verification

pGhU6 vector (provided by Merixtell Albrich-Jorda in Prague Institute of Molecular Genetics of the ASCR) was digested with the restriction enzymes XhoI and HpaI (New England Biolabs cat no. R01465 and R01055, respectively) overnight at room temperature. The digested plasmid DNA was run in 1.2% horizontal electrophoresis gel (Sigma life science cat no. A9539-500G) electrophoresis for 1:30 hours and the desired band was visualized and cut out under UV light imaging (Gene flash Syngene bio imagine). The digested plasmid was purified using a Gene jet extraction kit (Themofisher cat no K0691).

An Nme2 shRNA synthetic oligo made by annealing the following primers (Biomers.net GmbH biopolymers Factory) was obtained from Dr. Enrica Bach, Department of Hematology, University of Leipzig.

Forward	5'AACGAGAATAGAACGACCACCAGCCGTTTTGGCCACTGACTGACGGCTGGTG CGTTCTATTCTC-3'
Reverse	5'TCGAGAGAATAGAACGCACCAGCCGTCAGTCAGTGGCCAAAACGGCTGGTG GTCGTTCTATTCTCGTT-3'

The annealed oligonucleotide has one HpaI-compatible blunt end (New England BioLabs R01055)-and one XhoI-compatible TCGA overhang (New England BioLabs R01465). The oligonucleotide dsNme2 2µl (50ng/ul) was ligated to digested pGhU6 1µl (35ng/ul) using T4DNA ligase and T4 ligase buffer (New England BioLabs MO202S and BO202S, respectively) at 4°C overnight then on the next day the mixture was heated at 65 °C to deactivate the ligase reaction. The ligation mix was used to transform, chemically competent one shot TOP 10 E coli (Invitrogen Thermofisher cat no: C404010) according to the manufacturer's instructions. The transformation mix was spread onto LB agar AMP (Lysogeny Broth (LB), 1.5% agar containing 1:1000 of (100mg/ml) Ampicillin antibiotic (Carl Roth GmbH k0291+Go. K , Germany). After overnight incubation, 5 colonies were picked and expanded overnight in LB AMP liquid medium. Plasmid DNA minipreps were prepared using a Qiaprep spin mini prep kit (Qiagen, cat no 27104/27106) according to the manufacturer's instructions. The DNA concentration was determined by spectrophotometry (Theromscientific Nanodrop lite spectrophotmeter). The DNA sequencing was carried out in the Core Unit DNA Technologies, University of Leipzig Faculty of Medicine done using primers for the pGhU6 vector: psicoR tgc agg gga aag aat agt agac and LOK.1 5' hU6 promoter gac tat cat atg ctt acc gt (Biomers.net GmbH biopolymers Factory). Once the identity had been confirmed by sequencing (Fig 2-1), maxi prep DNA was prepared from a large scale culture (Qiagen plasmid plus maxi kit cat no 12963/12965, Germany) . The shNme and Empty vector DNAs were preserved at -20^{o}C for further use.


PGhU6
(7618) NotI
NsiI (3)
NdeI (238)
BsaAI - SnaBI (344)
NheI (595)
BmtI (599)
AfeI (600)
AgeI (604)
EGFP-N (662 .. 683)
EXFP-R (923 .. 942)
EGFP-C (1270 .. 1291)
BsrGI (1326)
EcoRI (1337)
AflIII - PciI (1390)
NspI (1394)
WPRE-R (1498 .. 1518)
PflMI (1686)
SacII (1948)
BbsI (1973)
PvuI (2054)
Bsu36I (2167)
Acc65I (2171)
KpnI (2175)
PvuII (2202)
BbsI (2273)
DraIII (2453)
BspEI (2549)
NspI (2747)
DrdI (2851)
pBR322ori-F (2884 .. 2903)
BseYI (3047)
BspHI (3463)
AhdI (3636)
FspI (3858)
AclI (3862)
PvuI (4006)
ScaI (4116)
Amp-R (4186 .. 4205)
AclI - XmnI (4235)
SspI (4440)
BspHI (4471)
BsrBI (4477)
pRS-marker (4603 .. 4622)
MfeI (4720)
NruI (4767)
MluI (4787)
SpeI (4808)
NdeI (5043)
BsaAI - SnaBI (5149)
NarI (5574)
SfoI (5575)
PluTI (5576)
BssHII (5578)
NruI* (5770)
XmnI (5781)
SapI (6039)
FseI (6087)
EcoNI (6107)
MfeI (6126)
BbvCI (6361)
BstAPI (6390)
BsmI (6531)
BsaXI (5755)
BsaXI (6785)
BstAPI - DraIII (6958)
PstI (6974)
PspOMI (7162)
ApaI (7166)
BsrBI (7168)
XbaI (7170)
SspI (7272)
mU6-F (7407 .. 7427)
HpaI (7488)
BstEII (7496)
PaeR7I - PspXI - XhoI (7503)
BfuAI - BspMI (7537)
loxP
KS primer
SSFFV promoter
Kozak sequence
EGFP
cPPT/CTS
RRE
5' LTR (truncated)
HIV-1 ψ
CMV promoter
CMV enhancer
AmpR promoter
AmpR
ori
5' LTR (truncated)
WPRE
Factor Xa site
KS primer
1000
2000
3000
4000
5000
6000
7000

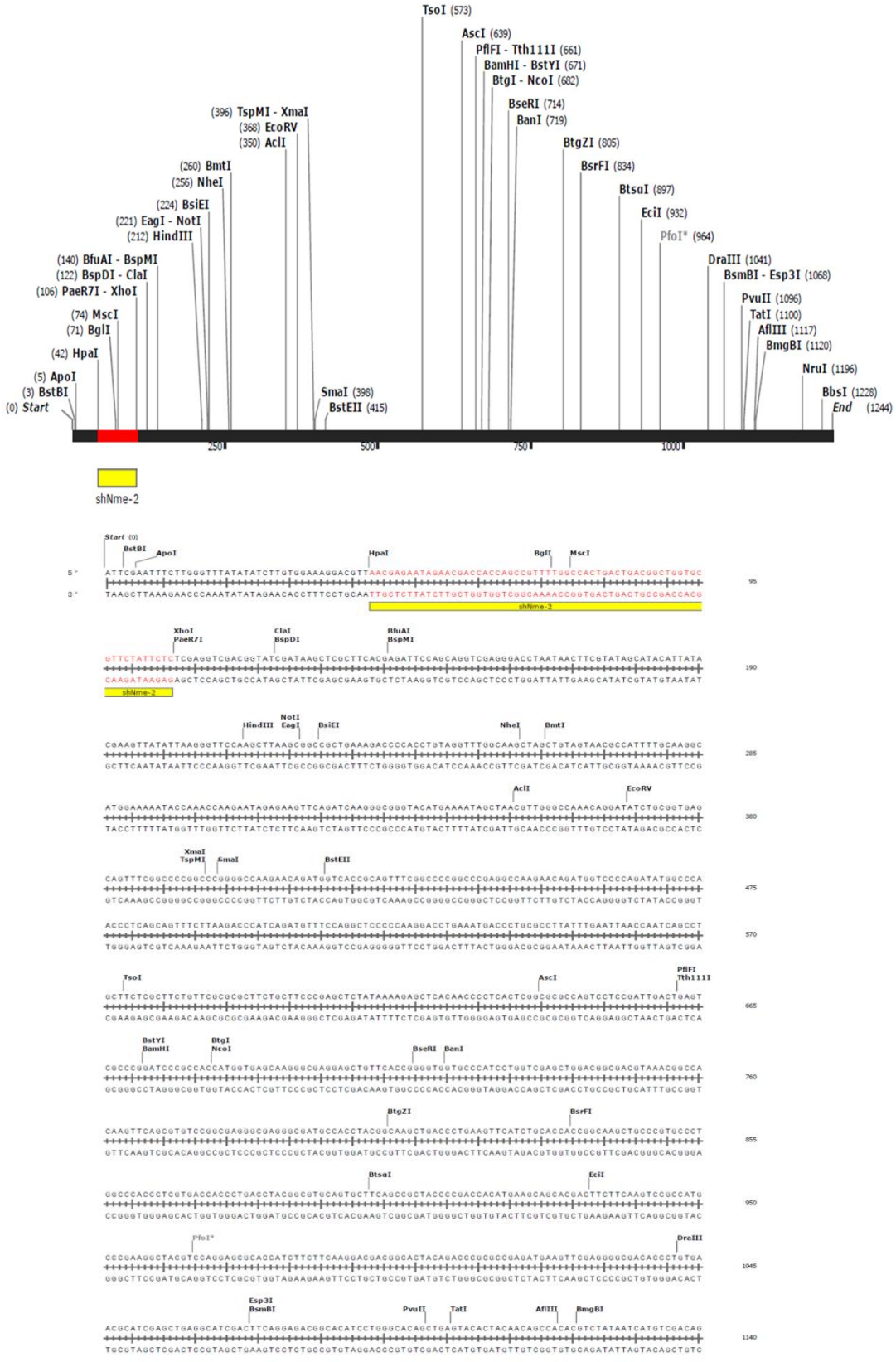


Fig 2-1 The pGhU6 vector map derived from Pll 3.7 vector and the area of shNme2 insert.

2.8 Lentivirus production and transduction

In principle, a lentivirus expresses reverse transcriptase, which converts the viral RNA to double stranded DNA, and integrase, which integrates this viral DNA into the host DNA. Once the viral DNA is integrated into the host DNA, it replicates through the host genome. Lentiviral transduction is an efficient method for the distribution of transgenes to dividing and non- dividing mammalian cells (Elegheert et al. 2018). In the current study, a 3rd generation packaging system was used with two separated plasmids, the Group Specific Antigen/ DNA polymerase (gag/pol) and the Vesicular Stomatitis Virus/glycoprotein G (VSV-G).

The lentiviral particles were produced in 293T fibroblast cells according to addgene protocol guideline (addgene 2016, 2019). The 293T cells (3.8×10^6) were seeded in a T75 flask in 15 ml Dulbecco's Modified Eagle's Medium DMEM , 10% v/v FBS and 4 mM L-alanyl-L-glutamine).The cells were incubated at 37 ℃, 5% CO_2 overnight then gently the media was aspirated and changed with 10ml fresh DMEM complete containing 25 µM cloroquine diphosphate and incubated for 5 hours. Later the mixture of transfection plasmids was prepared as follows : 12µg packaging vector gag/pol (addgene cat no 12260) +1.4 µg VSV-g (addgene cat no 12260) + 15µg the previously described construct shNme2 or the empty vec tor as control +1:1000 of 1mg/ml polyethylenimine PEI (Sigma Aldrich cat no 408727) in 2 ml Opti-MEM (Thermo-Fisher scientific cat no 31985070,USA). The ratio between PEI /Opti-MEM and the vectors was 3:1. Then, the mixture was incubated for 20 minutes at RT. Thereafter, the transfection mixture was mixed dropwise to the Lenti-X 293T packaging cells and incubated overnight. On the following day the media was aspirated and replaced with fresh 15 ml DMEM complete followed by an overnight incubation. Next, the virus was harvested at 48, 72, and 96 hours post transfection and the Green Fluorescent Protein GFP fluorescence was evaluated using the (Zeiss Vert.A1) microscope. Eventually, the collected virus supernatants were centrifuged at 500xg for 5 mintues to pellet any packaging cells that were collected during harvesting. The viral supernatants were stored in small aliquots at -80 ℃ freezer until the target transfection.

For target cell transfection, the standard polybrene (sigma cat no H268) spin centrifugation transfection protocol was used as it described by (addgene 2016, 2019). A pilot study was performed to optimize the best ratio of lentivirus and the media. The culture media were, IMDM, 10% HS, 10% cmIL3 for FDCP-Mix and RPMI, 10% FBS for K562. Polybrene 10µg/ml was added to both media . The ratio of 1:3 (virus:polybrene) produced the best transfection result for FDCP-Mix with 66% transfection efficiency for both the control empty vector and shNme2 construct (700µl virus + 300µl DMEM complete/ polybrene media + 1000µl DMEM complete/ polybrene media with $2x10^5$cell density) . The 6 well plates were centrifuged at 1200xg at 30 ^oC for 90 minutes and the control FDCP-Mix, shNme2 FDCP-Mix and control K562 were incubated overnight in biosafety level 2 S2 incubator at 37^oC 5%CO_2 . On the next day the culture media was changed for fresh media and the cells were returned to the incubator. The cells were checked for GFP expression daily using a fluorescence microscope

(Zeiss Vert.A1). As pGhU6 doesn't contain a selectable antibiotic resistance marker, the FDCP-Mix that expressed GFP were FACS sorted in the Core Unit Fluoreszenz–Technologien (BD FACSAria II SORP).

2.9 Gene expression analysis by (qRT-PCR)

For determination of the expression of Nme2 (NDPKB) mRNA, $1X10^6$ FDCP-Mix cells were lysed and RNA was extracted with the RNeasy Micro or Mini Kit (Qiagen cat no 74106 or 74004) according to the manufacturer's instructions. The RNA was eluted in a final volume of 10 µl, which was reverse transcribed into cDNA using the Thermofisher high capacity RNA to cDNA kit (cat no 4387406), in a final reaction volume 20µl (10µl RNA + 2µl 10x RT buffer + 0.8 µl 25x dNTP mix + 2µl 10x random primers + 3.2µl H_2O + 1µl RNase inhibitor + 1µl Multiscribe reverse transcriptase). The reverse transcriptase reaction was carried out for 10 minutes at 25^{o}C then 120 minutes at 37^{o}C, followed by a denaturation step for 5 minutes at 85^{o}C then cooled to 12^{o}C before transferring to storage at -20^{o}C.

The quantitative measurement of Nme2 transcript was carried out using a SYBRgreen-based real-time quantitative PCR (Thermo Fisher cat 4368577) run on an Applied Bioystems 7500 Real time PCR machine. For each reaction, 2µl of the cDNA product was used in a final reaction volume of 25µl (9.5 µl H_2O + 0.5µl each primer + 12.5µl SYBRgreen mastermix + 2µl cDNA). Following activation of the polymerase at 95^{o}C for 15 minutes, the PCR reactions were run for 45 cycles of 15 sec denaturation at 94^{o}C, 30 sec annealing at 63^{o}C, 35 sec extension at 72^{o}C. Product melting curves were determined by denaturation at 94^{o}C for 15 secs followed by 30 secs annealing at 60^{o}C and heating from 60^{o}C to 95^{o}C at 15 secs per degree.

The mRNA expression for each gene was determined by analysis of triplicate samples in all cases. The obtained C_t average (MW) of the gene under consideration (nme2) was normalized to that of the household gene (rplp0) to obtain the ΔC_t value. This was then compared to a control sample (e.g., untreated cell line or d0 of a time course) to determine how many times higher or lower the expression of a particular gene of a sample was compared to the control.

Calculations: $\Delta c_{T\ nme2} = MW\ c_{T\ nme2} – MW\ c_{T\ rplp0}$

$\Delta\Delta c_{T\ nme2} = \Delta c_{T\ nme2}$ Probe - $\Delta c_{T\ nme2}$ Control

X-fold Expression nme2 = $2^{-\Delta\Delta Ct}$ nme2

2.10. Flow cytometry analysis

2.10.1. Cell surface staining

Cells ($5x10^5$/ml) were washed with staining buffer (450ml distilled water, 50 ml 10X PBS Gibco PH7.4REF70011036, 2.5ml 0.5%FBS PAN Biotech GmbH cat no. P261109, 5ml 1% NaN_3). The cells then were centrifuged at 800xg for 10 minutes, the supernatant removed and the cells re-suspended in 100µl staining buffer. 10µl blocking reagent was added for 15

minutes. Then, in prelabelled FACs tubes 2µl of each required antibody was added. The antibodies used in this study were: Pacific blue IgG2b isotype control clone RTK453, Biolegend cat no.400627; Pacific blue anti- mouse CD 117(c-kit) clone 2B8, Biolegend cat no.105820; PE rat IgG2a isotype control clone RTK2758, Biolegend cat no. 400508; PE rat anti-mouse CD71 clone R17217, Biolegend cat no. 113808; Alexa fluor647 rat IgG2a isotype control clone R35-95, BD cat no557690 and Alexafluor 647 anti-mouse Gr-1 clone RB6-8C5, Biolegend cat no 108418. The cell suspensions were incubated with antibodies for 15 minutes at room temperature in the dark. One ml of staining buffer was added and the FACs tubes were centrifuged 800xg for 5 minutes at 4°C. The supernatant was then removed carefully and the cells were re-suspended in 500µl staining buffer, then stored on ice until measurement. FACS analyses were carried out using a FACS Verse.

2.10.2 Apoptosis analysis

For discrimination of apoptotic, necrotic and dead cells both annexin V-FITC and propidium iodide were used. The apoptotic cells stain positively for Annexin V-FITC that binds to phosphotidylserine but are negative for propidium iodide. Dead cells stain with Annexin V-FITC and PI, whereas viable cells are negative for both Annexin V-FITC and PI. Cells were stained according to Miltenyi Biotec protocol (Order no. 130-092-052). Total cells ($5x10^5$) were washed with 1X Binding buffer and centrifuged at 300Xg for 10 minutes and the pellets were suspended in 100µl 1X Binding buffer. Afterwards, 5µl of Annexin V-FITC were added and incubated for 15 minutes in the dark at RT. Next, the cells were washed by 1 ml 1X Binding buffer and centrifuged as previously mentioned. Subsequently, the cell pellets were re-suspended in 250ul 1X Binding buffer to which 2,5µl of (100µg/ml) PI were added immediately prior to analysis by flow cytometry (FACS Verse).

2.10.3 Cell cycle analysis (NAM project)

Cells were cultured in medium containing 0.03, 1, 5, or 10 mM NAM for 4 days. After the incubation period cells were centrifuged at 800xg for 10 minutes and the supernatant was removed. The cell pellets were re-suspended in 0.5 ml PBS/1mM EDTA. Cells were fixed overnight at 4°C with 1 ml cold 70% ethanol and stored at 4^oC until analysis. For analysis of cell cycle, the cells were spun down at RT 800xg for 5 minutes, then the supernatant was carefully removed and the cells were re-suspended in 250µl PBS/1mM EDTA/RNase (10mg/ml) in a pre-labelled FACS tube. Finally, 1:100 of PI (1µg/µl stock solution) was added and mixed well then the cell cycle analysis was carried out with FACs verse.

2.11 ATP Bioluminescence assay

For ATP measurement, the ATP Bioluminescence assay Kit HS II (ROCHE cat no 11699709001, Germany) was used.

Principally, in this technique, the main reagent (luciferase) from Photinus pyralis (American firefly) catalyses the following reaction:

$$ATP + D\text{-luciferin} + O_2 \rightarrow \text{oxyluciferin} + PPi + AMP + CO_2 + \text{light}$$

The resulting green light has an emission maximum at 562 nm. Given saturating amounts of luciferase and D-luciferin, the light output is directly proportional to the ATP concentration. In the current study, the cells ($2x10^7$) were lysed with 200µl lysis reagent. Thereafter, the cells were incubated for 5 minutes at RT followed by centrifugation at 10,000xg for 30 seconds. The supernatant was then transferred to a fresh microcentrifuge tube and kept on ice until ATP measurement. Next, 100µl from the sample supernatant or the ATP standard controls were transferred to the measuring tubes. Lastly, the tubes were uploaded to the luminuteometer LUMAT LB 9507 from (BETHOLD TECHNOLOGIES GmbH&Co.KG, Germany) to ATP The concentration was calculated using a log-log plot of the standard curve data.

2.12 Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analysis was performed w using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. Differences with $p< 0.05$ were considered statistically significant.

3. Results

Section I : Metabolic effects on signaling pathways in haematopoietic progenitor cells (Figures 3-1 to 3-6)

3.1 Restricting the glucose available to FDCP-Mix cells affects the balance of JAK-STAT and Ras-MAPK signaling from the IL-3 receptor

Preliminary experiments were performed to see whether establishing a low energy environment by simply lowering the amount of glucose in the culture medium affects the JAK-STAT (self-renewal) and MEK-ERK (differentiation) responses to IL-3 to the same extent, or whether reducing glucose supply affects the balance between these two pathways. FDCP-Mix cells maintained under standard self-renewal conditions in the presence of high IL-3 were washed and transferred for 2 hours to IL-3-free medium containing either 5mM or 0.1mM glucose. At the end of this period, IL-3 was added back for a period of 10 minutes after which the cells were fixed and subject to phosflow analysis to assess the pSTAT5 and pERK1/2 responses. Test cultures were compared to identical control cultures which had not been re-stimulated with IL-3 (Fig 3-1). Consistent with the expected decrease in intracellular ATP levels, glucose restriction led to a sharp reduction in the pERK1/2 response. Surprisingly, the pSTAT5 response in the same cells was not merely maintained but was actually increased significantly following glucose restriction. This shows that the balance between signals associated with self-renewal and differentiation can indeed be affected by simple changes in the metabolic environment of the cells.

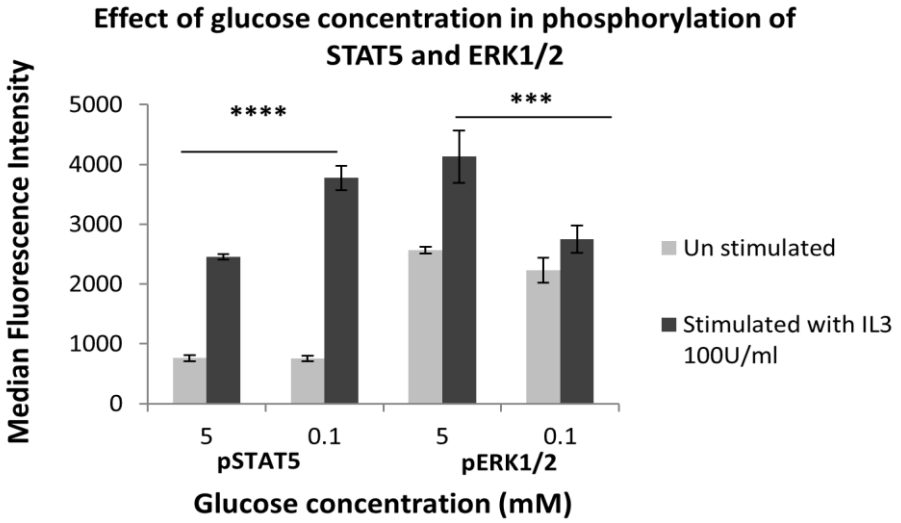


Fig 3-1. Reducing the glucose concentration from 5mM to 0.1mM disables the pERK1/2 response, but actually increases the pSTAT5 response to IL-3**.** Results are expressed as the median fluorescence intensity of phosphorylated STAT5 or ERK1/2 from biological triplicates (n=3; ***, p<0.001 ; ****, p<0.0000. Data expressed as the mean± SD).

The dependence of signaling on metabolic pathway activity was then analyzed in more detail using two inhibitors of cellular metabolism: 2-deoxyglucose (2DG), a competitive inhibitor of phosphoglucose isomerase that effectively blocks glycolysis at the second step; and rotenone, which inhibits complex I of the mitochondrial electron transport chain and thus blocks oxidative phosphorylation.

FDCP-Mix cells were first incubated in various concentrations of inhibitors (for 2DG 1mM, 5mM, 20mM and for rotenone 0.5uM, 1uM and 2uM) in order to determinate the maximum concentrations at which viability remained above 50% following overnight incubation. Using the chosen concentrations (1mM for 2DG and 1uM for rotenone), fresh FDCP-Mix were then incubated for 2 hs and 4 hs before measuring viability and intracellular ATP concentration. This showed that the maximal reduction in intracellular ATP concentration was achieved after 2 hours, at which time viability remained above 75% (Fig 3-3).

On the basis of these preliminary tests, inhibitors were then included in the 2 hour period of IL-3 deprivation immediately preceding phosflow analysis, in medium containing 5mM glucose. Since the kinetics of JAK-STAT and MEK-ERK signaling may differ, phosflow analyses were performed on cells fixed at 0, 5, 15 and 30 minutes after re-addition of IL-3 in order to be able to distinguish between reductions and delays in activity. As presented in (Fig 3-2), the highest levels of both pERK1/2 and pSTAT5 were detected under standard, non-inhibited culture conditions at the earliest time point tested of 5 minutes post-stimulation. Consistent with previous studies in haematopoietic stem and progenitor cells (Kalaitzidis and Neel 2008), pERK1/2 increased transiently, with levels returning to near-background within 30 minutes, while pSTAT5 persisted at a high level up to the 30 minutes time point.

Pretreatment with 2-DG to restrict glycolytic flux reduced the pERK1/2 signal strength without there being an obvious shift in the kinetics. A similar, but less marked effect was seen following the inhibition of mitochondrial respiration with rotenone. As expected, the combination of both inhibitors effectively blocked the MEK–ERK response to IL-3 completely, consistent with an inability to produce sufficient ATP to support kinase activity. This pattern suggests that the energy used for MEK-ERK signaling is derived primarily from glycolysis, with mitochondrial respiration playing a supportive role.

The pSTAT5 response to IL-3 differed from the ERK1/2 response in two important aspects: Firstly, acute phosphorylation of STAT5 was strongly dependent on mitochondrial function rather than on glycolysis. This difference is clearly not consistent with the activity of both pathways being dependent on the same pool of free ATP. Secondly, while the combination of rotenone and 2DG effectively prevented short term pERK1/2 signaling entirely, there remained a distinct pSTAT5 response in the same cells.

The increase in pSTAT5 levels seen at later time points post-stimulation in rotenone blocked cells is likely to reflect a compensatory induction of glycolysis, since it does not occur in the presence of 2-DG introduced either during the 2 hour period of IL-3 starvation (Fig 3-2) or together with the IL-3 at the time of re-stimulation.

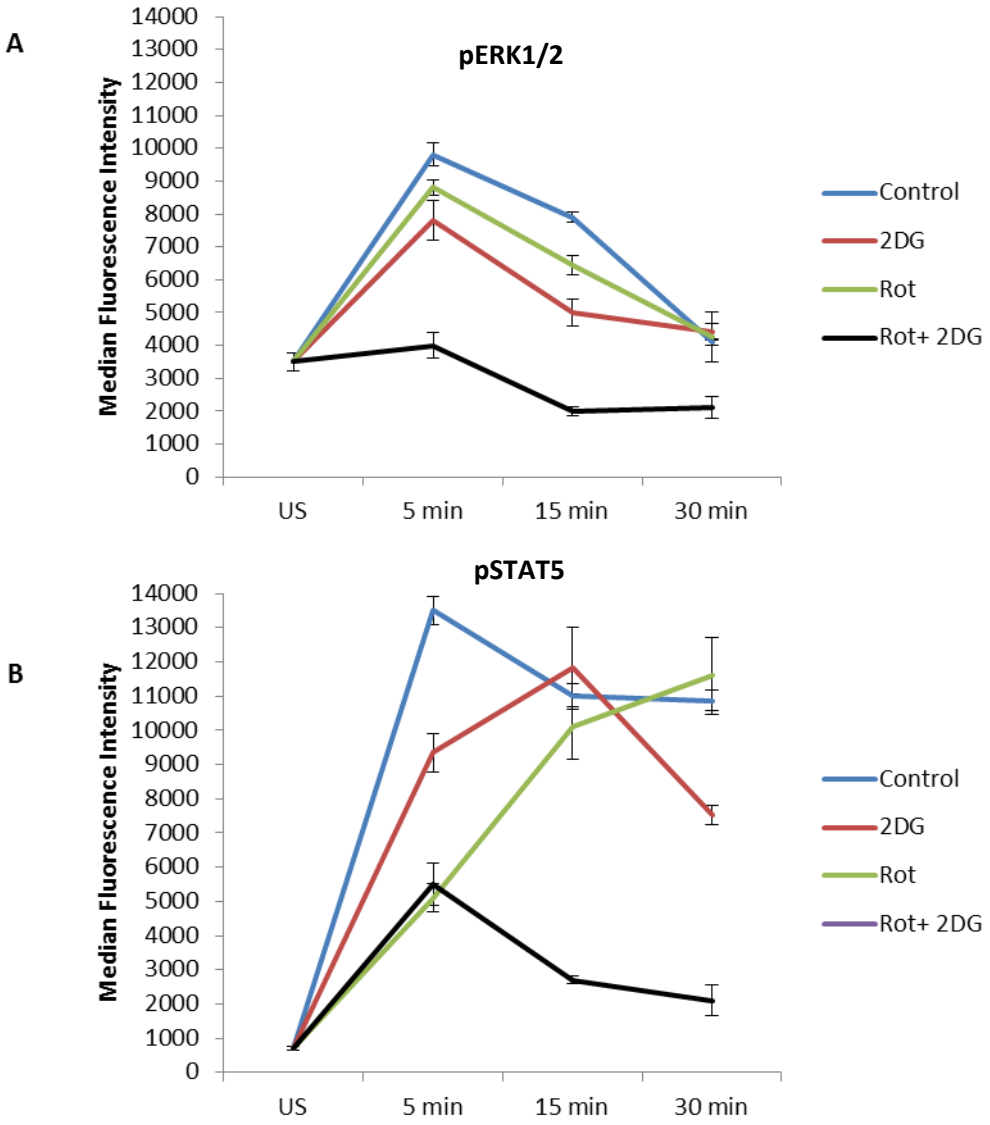


Fig 3-2 A and B The acute pERK1/2 response to IL-3 (Ras-MAPK pathway) can use energy from glycolysis or mitochondrial respiration. The acute pSTAT5 response to IL-3 (JAK-STAT pathway) appears to be particularly dependent on energy from mitochondria. When both glycolysis and oxidative phosphorylation are blocked, the pERK1/2 response is reduced to zero, while de novo STAT5 phosphorylation is still possible. Note: at the 5 minutes time point, pSTAT5 levels in Rot inhibited cells were significantly lower than in 2DG-inhibited cells ($p = 0.005$, while pERK1/2 levels were similar ($p = 1.000$). Data expressed as the mean± SE.

3.2 IL-3 dependent STAT5 phosphorylation does not correlate with free ATP levels

The ability of the JAK-STAT pathway to respond under conditions that effectively inactivate MEK -ERK signaling could simply reflect a requirement for higher levels of ATP to complete the longer MEK-ERK kinase cascade. However, the observation that the pERK1/2 and pSTAT5 responses are differentially reliant on glycolysis and mitochondrial electron transport suggests that there may be qualitative rather than quantitative differences in the energy use between the two pathways.

In order to correlate changes in signaling with ATP levels, free ATP was measured in extracts of FDCP-Mix cells grown in nutrient rich medium and pre-treated for 2 hours with 2-DG, rotenone, or both. The results (Fig 3-3) confirmed that inhibition of glycolysis reduces ATP levels more drastically than does inhibition of mitochondrial electron transport.

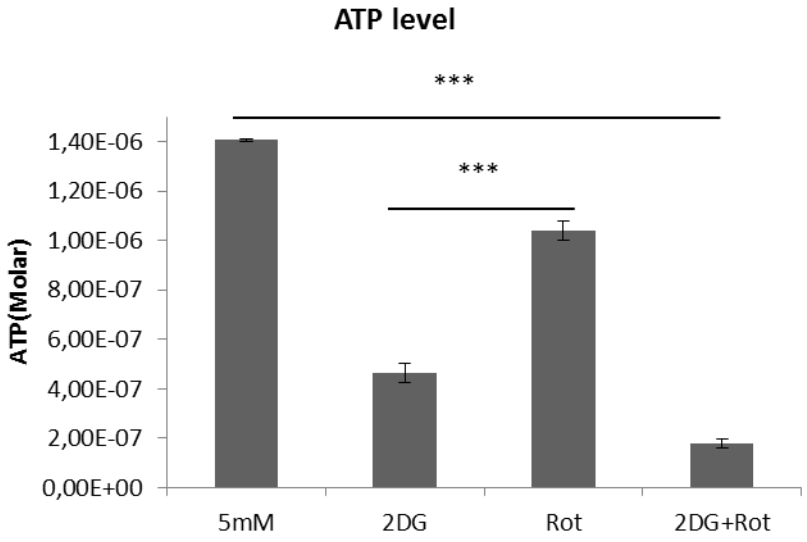


Fig 3-3 Levels of ATP in FDCP-Mix cells exposed for 2 hours to metabolic inhibitors. FDCP-Mix were exposed to 2 deoxyglucose (2DG), rotenone (Rot) or a combination of both (n=3 ***, p<0.001. Data expressed as the mean± SD).

This confirmed that while the pattern of Ras-MAPK pathway activity follows closely that of free ATP, activity of the JAK-STAT pathway appears to depend on something other than simple ATP concentration.

3.3 STAT5 phosphorylation at low-energy is dependent on the nucleoside diphosphate kinase Nme2

The maintenance of ATP-dependent kinase activity at low ATP concentration suggests the possible involvement of a nucleoside diphosphate kinases (NDPK) activity. NDPKs transfer the gamma phosphate from nucleoside triphosphates to nucleoside diphosphates. The transfer from ATP to a non-adenosine NDP to generate NTP and ADP is generally considered to be the forward reaction. However, this reaction is reversible so that the NDP kinases may also take the gamma phosphate from NTPs to regenerate ATP from ADP (Stahl et al. 1991). The NDP kinase Nme2 has previously been reported to be overexpressed at the protein level in chronic myeloid leukemia, in which Nme2 protein levels correlate closely to the activity of BCR/ABL (Tschiedel et al. 2012). Furthermore, the maintenance of Nme2 mRNA but not of Nme1 mRNA levels in starved bone marrow cells has been found to correlate with prognosis in acute myeloid leukemia (Bach et al., 2012). Since Nme proteins are known to bind directly to a variety of targets (Dexheimer et al. 2009; Postel et al. 2009; Postel et al. 1993), it is possible that Nme2 is involved in the maintenance of JAK-STAT signaling at low ATP levels by recharging local ADP at the expense of other NTPs. To test for a possible requirement for Nme2, a lentiviral shRNA vector targeting Nme2 was constructed and used in comparison to

an empty vector control to transfect FDCP-Mix cells. Under standard, high energy culture conditions, quantitative real time PCR measurement showed a modest (though significant) difference in Nme2 mRNA levels between shRNA-expressing and control cells. However, glucose restriction resulted in both an increase in Nme2 mRNA level in vector-control cells and a reduction in Nme2 shRNA-expressing cells, suggesting that there may be an altered turnover of Nme2 mRNA under energy-limiting conditions.

Functional comparisons of shNme2 and control-transfected populations in terms of proliferation rate, colony-forming capacity and differentiation potential revealed marked differences, despite the relatively small difference in Nme2 mRNA. Population doubling time over the first 3 passages was reduced from 26.9 hrs in the control to 55.4 hrs in the Nme2 knock-down cells (Fig 3-4B), while colony forming capacity was reduced by fully 10 fold (Fig 3-4C) and the erythroid differentiation response was impaired (Fig3-4D).

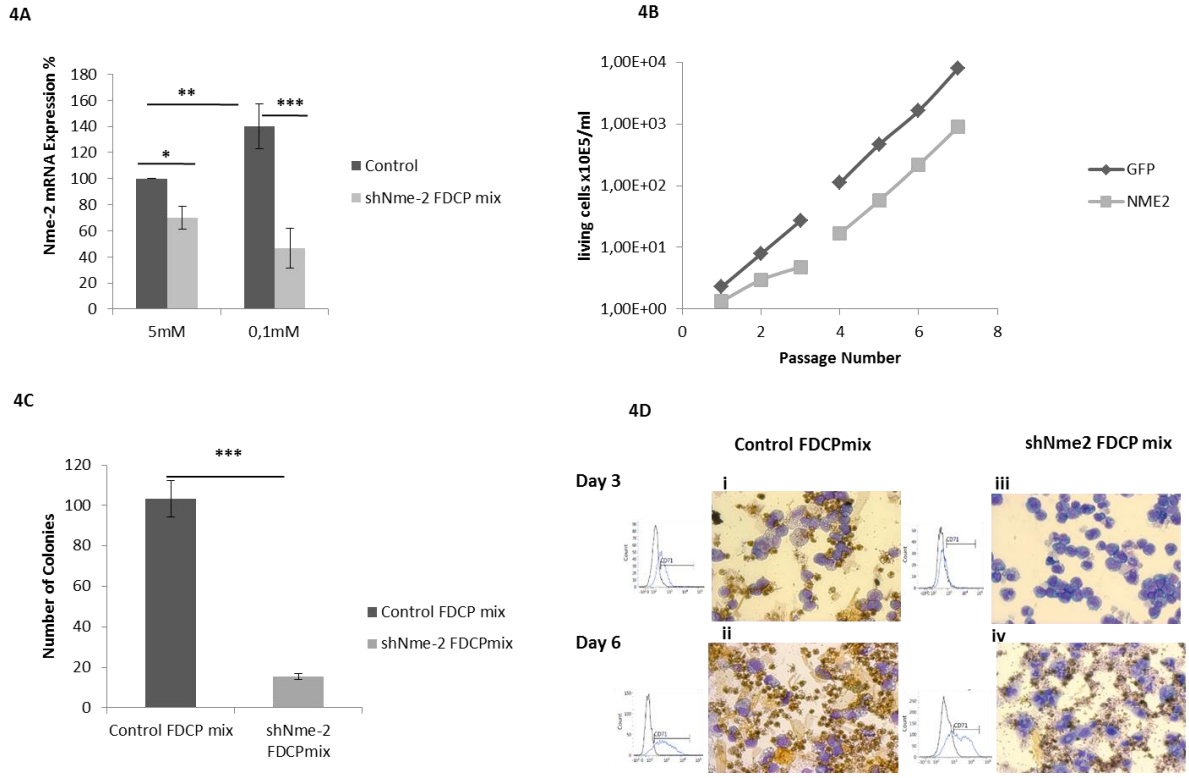


Fig3-4. The effects of shRNA-mediated Nme2 knockdown in FDCP-Mix cells **A.** Expression of Nme2 mRNA was significantly reduced in the knocked down haemo-progenitor cells (n=3 ;*,$p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data expressed as the mean± SD). **B.** Reducing of doubling time in knockdown cells up to third passage. **C.** Colony formation was significantly reduced in knockdown cells (n=3;*** $p < 0.0001$. Data expressed as the mean± SD). **D.** Normal haematopoiesis with a broad spectrum of myeloid and erythroid progenitors (left) in control transfected cells compared to the delayed erythroid differentiation seen in Nme2 knockdown cells (right).

Since Nme2 knockout mice show a defect in erythropoiesis (Postel et al. 2009), I also compared the differentiation response of control and shNme2 transfected cells by transferring the FDCP-Mix cells to media supporting both myeloid and erythroid differentiation. As can be seen in (Fig 3-4D), the erythroid differentiation of Nme2 knockdown cells was delayed in comparison to controls, with early differentiation in the

Nme2 knockdown cultures being predominantly myeloid and both CD71 expression and haemoglobin staining appearing much later than in the controls.

Consistent with the slower proliferation of Nme2 shRNA-expressing cells compared to empty vector controls, the shRNA-expressing populations used for the experiments described above tended to accumulate GFP-ve cells during culture. For this reason, signaling response was assessed in freshly sorted populations of GFP+ve cells (Fig 3-5). Under standard, high energy conditions, there was no difference between shRNA- and control vector-transfected cells in terms of the degree of IL-3-dependent STAT5 phosphorylation. However, this changed under the low energy conditions imposed by pre-treatment with 2DG and rotenone. Here, the majority of control cells maintained the capacity to mount a robust STAT5 response to IL-3 as noted above, while the Nme2 shRNA-transfected population had lost this ability, showing that Nme2 is indeed required for IL-3-dependent STAT5 phosphorylation under low-energy conditions.

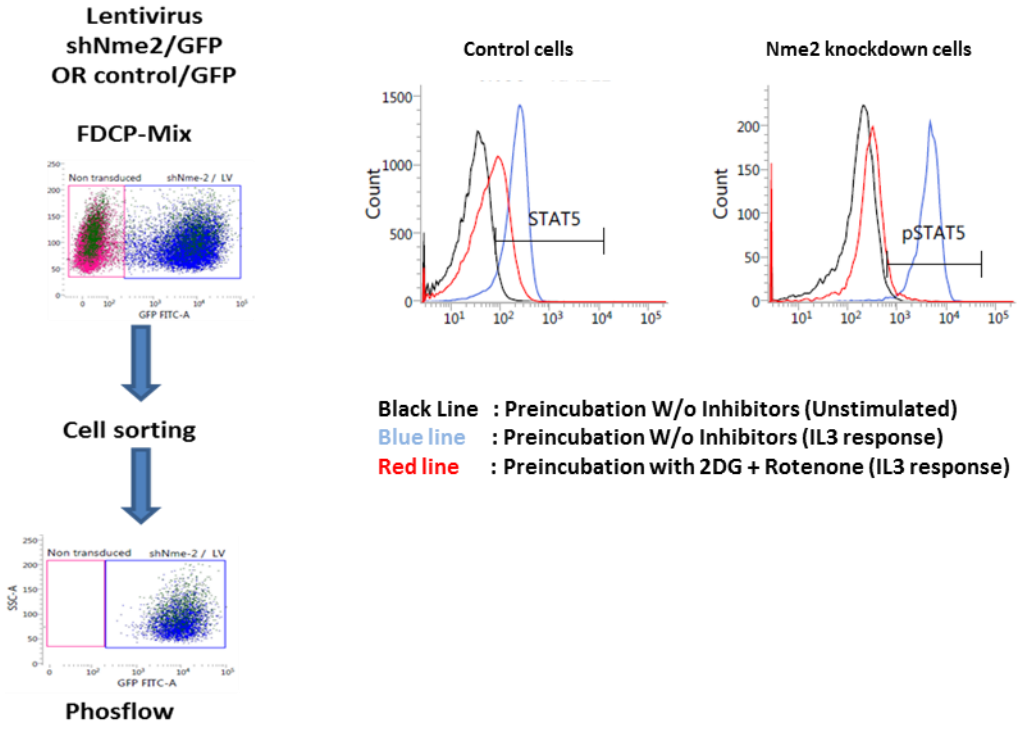


Fig 3.5. The effects of metabolic inhibitors on JAK-STAT signaling in Nme2 knockdown FDCP-Mix. The FACS plots on the left demonstrate the purity of FACS sorted, GFP+ve population used in the Phosflow analysis presented on the right. The figure shows a representative result from 3 independent experiments.

3.4 Pilot study in Primary UCB (Figures 3-6)

The studies with FDCP-Mix cells suggest that metabolic environment and metabolic activity may indeed influence the balance of signaling in haematopoietic stem and progenitor cells. In order to validate these findings in primary cells, the umbilical cord blood MNCs were cultured in serum free IMDM medium (contain SCF, IL3, TPO) for 2 days then the cell incubated with or without metabolic inhibitors for 2 hours in growth factor depleted medium.

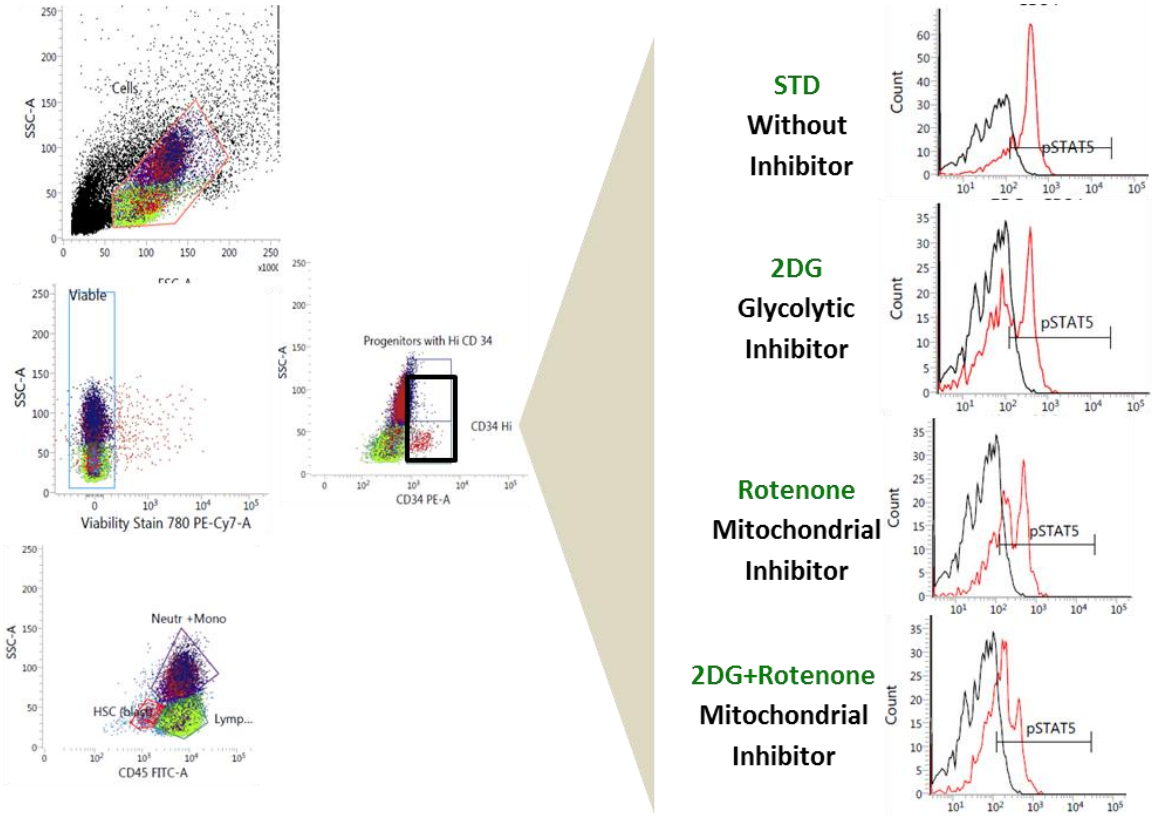


Fig 3-6 The effects of metabolic inhibitors on JAK-STAT signaling in UCB. The FACs plot on the left shows the gating strategy for CD34+ve and on right the pSTAT5 signaling. The figure shows a representative result from 3 independent experiments. Sub population of UCB CD34+ve capable to provoke STAT5 signaling under low energy condition (2DG+ROT).

To check the STAT5 response to IL3 under different metabolic stress, the cells were stimulated with human IL3 for 10 minutes prior to phosflow intracellular staining. A sub-population of UCB CD34+ve cells even in the energy restricted conditions were able to initiate STAT5 signaling. These preliminary results support the previous finding with the FDCP-Mix system. Due to the incompatibility of CD38 and CD133 markers with the phosflow technique, it was not possible to identify further the sub population.

Section II : Effects of medium osmolarity on the proliferation, clonogenicity and differentiation capacity on FDCP-Mix cells. (Figure 3-7)

3.5. Raising the osmolarity with mannitol does not have the same effect as raising the salt concentration

Previous work has shown sodium chloride (NaCl) supplementation of growth medium to decrease proliferation rate and increase the proportion of colony-forming cells in both FDCP-Mix and primary CD34+ cell cultures (data not shown). To determine whether the observed effects result from changes in salt concentration or in osmolarity, FDCP-Mix cells were cultured in standard medium (320 mOsm/L) or in medium that had been increased to 360 mOsm/L using sodium chloride, potassium chloride or the metabolically inert sugar mannitol. While salt supplementation decreased proliferation rate and increased colony forming activity, confirming previous observations, the supplementation of mannitol to the same osmolarity had no such effect (Fig 3-7A). This demonstrates that the positive effects of salt-adjusted osmolarity on the maintenance of haematopoietic stem and progenitor cells (Couthino et al., 1993) is most likely due to the effects of the salt, rather than to the osmolarity.

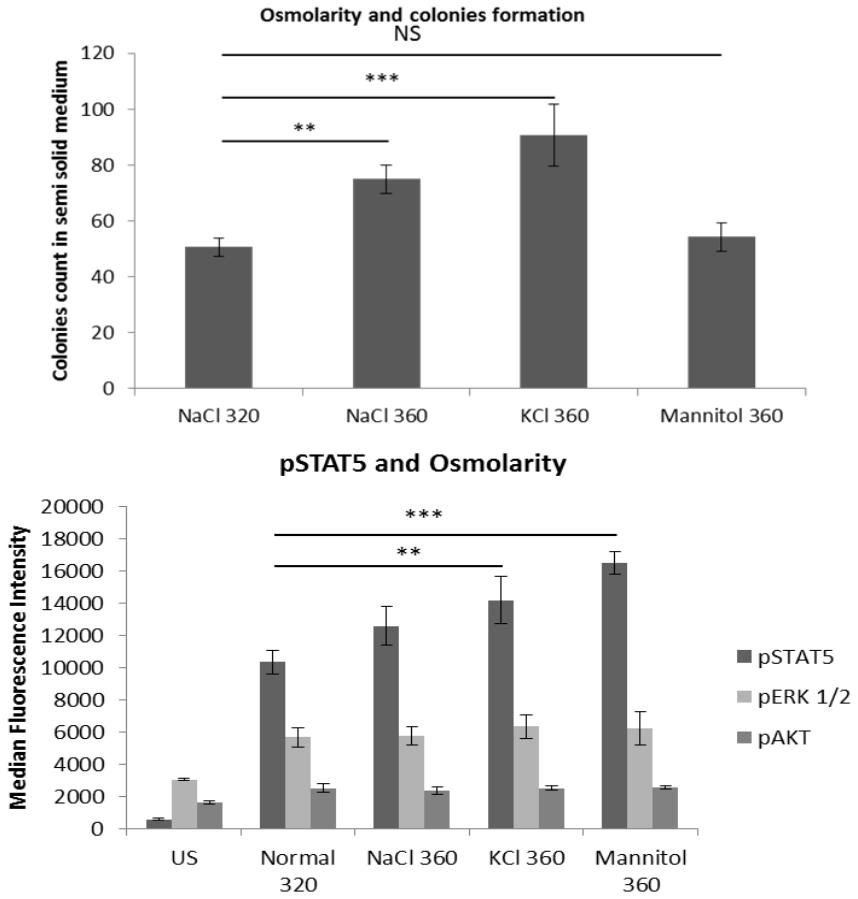


Fig 3-7 A Increased colony formation depends on salt supplementation rather than increased in osmolarity **B.** JAK-STAT activity is increased with salt supplemented media and with mannitol treatment. (n=3 ;**, $p < 0.01$; ***, $p < 0.001$; NS, not significant. Data expressed as the mean± SD).

Given that increasing salt concentration, but not osmolarity, increases the proportion of early, colony-forming progenitors in culture, it was of interest to test whether or not this is reflected by an effect on the balance of signaling pathways. To examine this, FDCP-Mix cells were cultured for 2 days in standard medium (320mOsm/kg) and in medium in which the osmolarity had been adjusted to 360 mOsm/kg using NaCl, KCl or mannitol as described above. Following a 2hr period of IL-3 starvation, the response of the JAK-STAT, Ras-MAPK and P-I3K–Akt signaling pathways were compared by phosflow analysis of pSTAT5, pERK1/2 and pAkt 15 minutes after re-stimulation with IL-3. The result, shown in (Fig 3-7B), shows that JAK-STAT activity is indeed selectively increased in the salt-supplemented media in which the frequency of colony-forming cells was higher. However, the JAK-STAT activity was increased still more by supplementation with mannitol, which had been shown to have no effect on the frequency of colony forming cells. Increasing osmolarity using either mannitol or salt therefore increases the JAK-STAT response relative to that of Ras-MAPK or PI-3-K-Akt pathways, but this alone is not sufficient to increase the frequency of colony forming cells in the culture.

Section III : The Effect of nicotinamide on the self-renewal and differentiation of haematopoietic progenitor cells (Figures 3-8A-G)

3.6 Nicotinamide slows proliferation while increasing apoptosis, necrosis and myeloid differentiation.

Nicotinamide can increase the amount of NAD+ and affect a variety of cellular processes (see Fig 1-3). NAM has been previously reported both to support granulocyte differentiation (Skokowa et al. 2009) and to enrich progenitor cells. It was therefore of interest to determine the effects of NAM in the FDCP-Mix system. As an initial test, cells were cultured in standard self-renewal conditions (high IL3 and horse serum, 0.03mM NAM) and in the same medium supplemented with extra NAM to 1mM, 5mM and 10mM. Cell number and viability were assessed every 2 days. FDCP-Mix cultured in differentiation medium (low IL-3, GM-CSF, G-CSF, fetal bovine serum and 0.03mM NAM) were included as a control. NAM was found to result in a concentration-dependent reduction in the overall rate of proliferation (Fig 3-8A). The fractions of viable, apoptotic and necrotic cells in the self-renewal cultures containing 0.03, 1 or 5mM NAM were assessed by FACS (Fig 3-8B). Consistent with the reduced proliferation, the FACS analysis revealed a concentration dependent increase in the frequency of necrotic (Annexin^{+}, PI^{+}) cells, indicating toxicity of NAM at high concentrations. However, the frequency of apoptotic cells (Annexin^{+}, PI^{-}) did not match that of necrotic cells. In particular, there was a peak in apoptosis at the 6 day time point in self-renewal cultures exposed to just 1mM NAM.

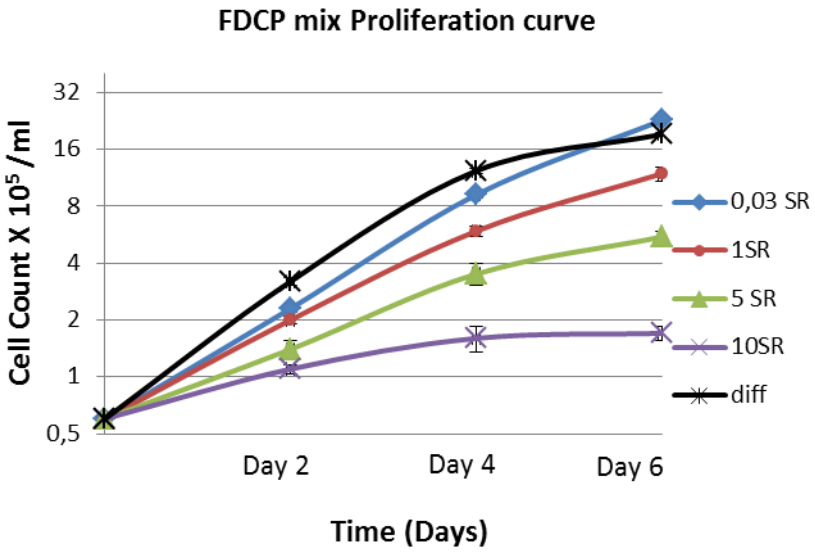


Fig 3-8A. shows the effect of NAM on cell proliferation. NAM reduce the overall rate of proliferation in a concentration-dependent manner. (n=3 $p < 0.001$. Data expressed as the mean± SD ;*SR: Self renewal; diff : Differentiation medium).

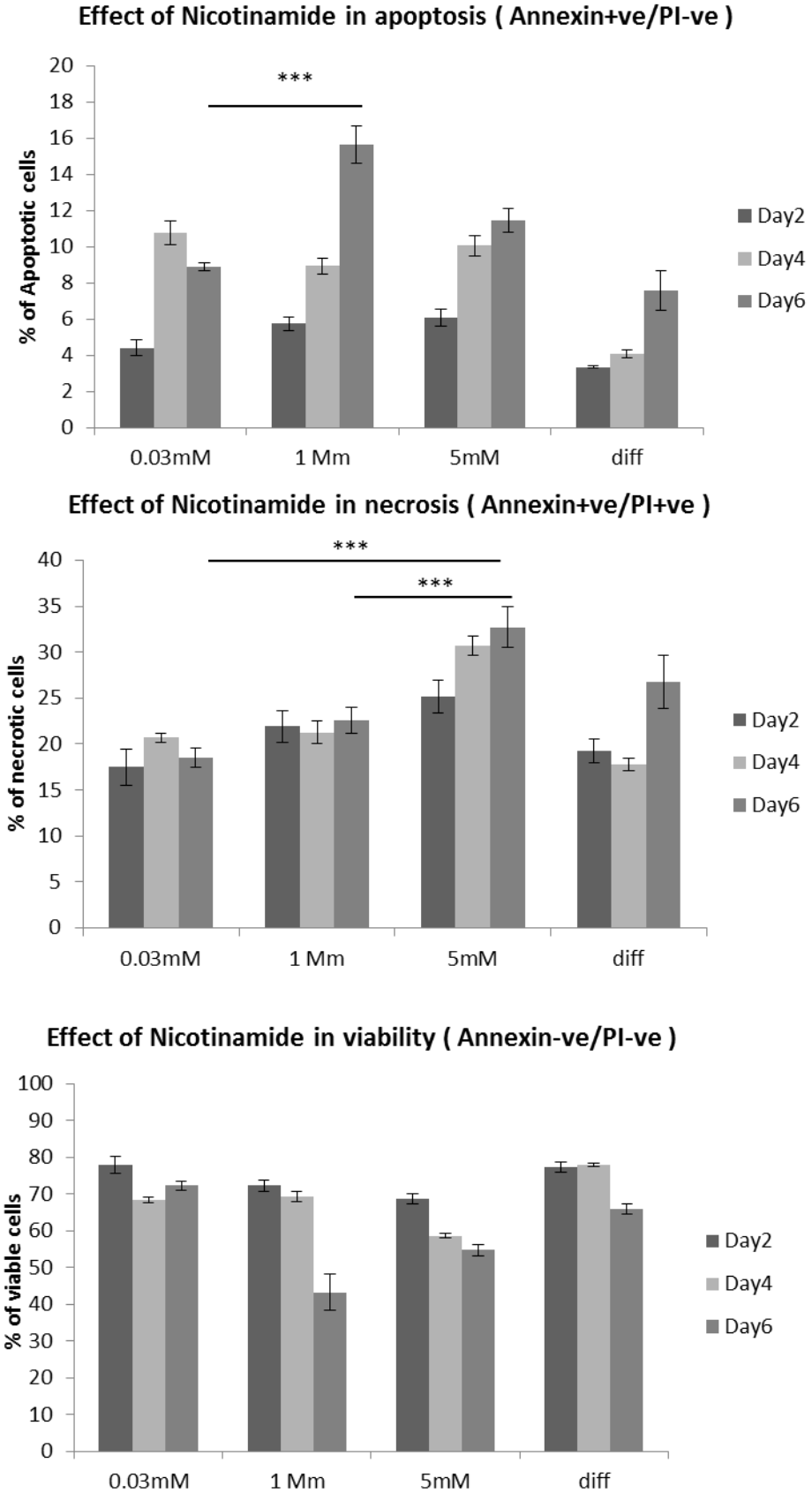


Fig 3-8B shows the effect of NAM on cell viability . NAM enhance the apoptosis at 1mM (n=3 $p < 0.001$. Data expressed as the mean± SD) and increase the toxicity (Necrosis) above 5mM.

Morphological examination of cytospin and stained cells from 4 day cultures (Fig 3-8C). showed maturing monocytes and granulocytes in cultures performed in the presence of 1mM NAM, despite the absence of added myeloid growth factors. These cells were not seen under non-supplemented self-renewal conditions (0.03mM NAM) and scattered in the presence of 5mM NAM, in which the cells were extensively vacuolated, consistent with NAM cytotoxity at this concentration. NAM therefore appears to support the rapid generation of morphologically mature myeloid cells in the absence of external myeloid growth factors GM-CSF or G-CSF or M-CSF.

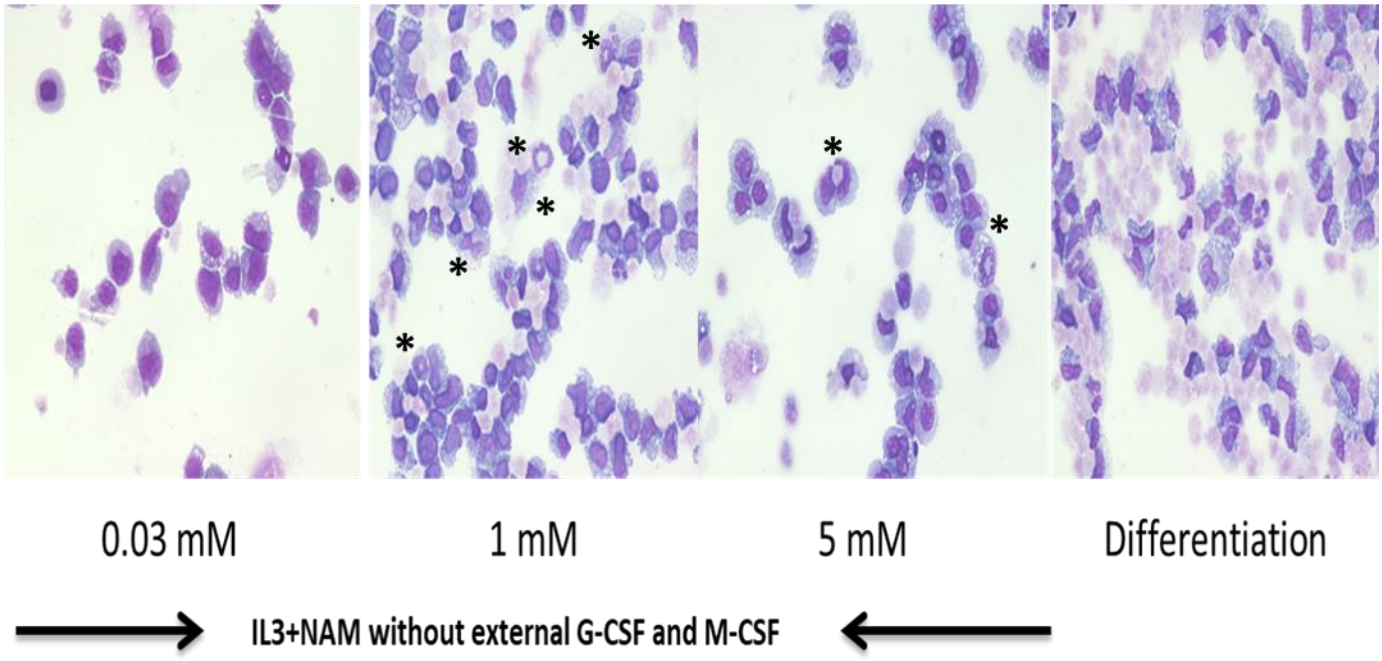


Fig 3-8C show the effect of NAM on cells morphology x60 oil. ✱,cells with myeloid morphology.

3.7 NAM reduces the proportion of cycling cells

The reduced proliferation rate of the FDCP-Mix population seen above could be a result of increased cell death and/or an effect of NAM on the cell cycle (Wang et al. 2018). Specifically, it may be predicted that the differentiation seen in the presence of 1mM NAM is associated with a reduction in cycling, since cell maturation usually occurs after proliferation has stopped. To test this, FDCP-Mix cells were cultured in self-renewal conditions for 4 days with different NAM concentrations, then after washing step with 1x PBS and fixing, the cells were stained with PI shown in (Fig 3-8D), the 1mM NAM concentration strongly reduced the proportion of cells in the S/G2/M phases of the cell cycle, and increased the proportion of sub-G1 events, which most probably correspond to cells undergoing apoptosis or necrosis. Higher NAM concentrations resulted in progressively fewer cells in the S/G2/M phase of the cycle. However, the major effect on cell cycle was already seen at the 1mM concentration. This pattern suggests that 1mM NAM has a major effect on cell cycle, while inducing differentiation and apoptosis, while higher concentrations of NAM are increasingly cytotoxic. The shift in the subG1 peak at higher NAM concentrations suggests increasing DNA degradation (Fig 3-8D FACs plot).

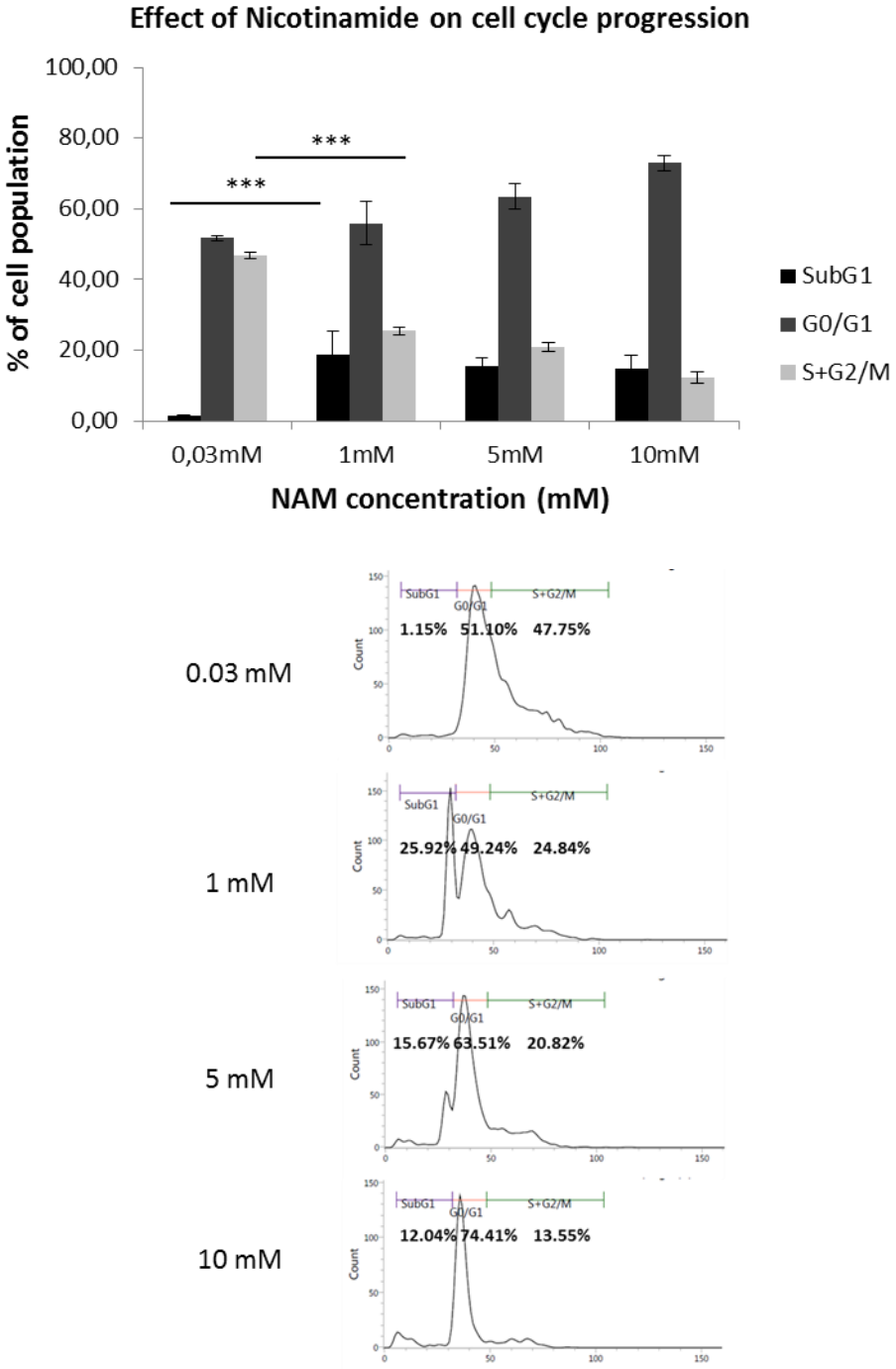


Fig 3-8D shows the effect of NAM on cell cycle. The lower figure shows representative FACs plot from 3 (0.03mM,1mM and 5mM) or 2 (10mM) independent experiments. NAM concentration strongly reduced the proportion of cells in the S/G2/M phases of the cell cycle, and increased the proportion of sub-G1 events. (n=3 ;**, p = 0.002; ***, p = 0.000. Data expressed as the mean± SD).

3.8 NAM reduce the erythroid and early progenitors markers without alteration in granulocytic markers

The presence of morphologically distinct granulocytes cells in FDCP-Mix cultures treated with NAM but no myeloid growth factors made it interesting to examine the expression of surface markers to see whether these are altered accordingly. Therefore, the cells were cultured in self-renewal medium with different NAM concentrations with standard

differentiation medium being included as a control. Surface markers were examined by FACS every on days 2, 4 and 6 of culture.

The Gr1 antigen is present on granulocytes and was increased accordingly under differentiation conditions peaking at day 4. The following reduction by day 6 is due to death of short lived granulocytes. Surprisingly, Gr1 expression in the cultures supplemented with 1mM NAM was only slightly (and not significantly) above the self-renewal control at day 4 and there was no increased expression of GR1 detectable at day 2 or day 6. This suggests that the morphological myeloid differentiation seen in the absence of added growth factors is not accompanied by the increased expression of GR1 typical of cells differentiating in the presence of added myeloid growth factors.

As can be seen in (Fig 3-8E), NAM supplementation did reduce the frequency of cells expressing the stem and progenitor marker c-Kit (CD117, stem cell factor receptor, expressed on stem and early progenitor cells) as well as the proportion expressing CD71 (transferrin receptor, expressed predominantly on early erythroid cells) in comparison to cells maintained in standard self-renewal conditions. The modest decrease in c-Kit and CD71 expression even under self-renewal conditions seen during the later stages of culture are typical of FDCP-Mix cells that reach a critical cell density. These densities were not reached in the NAM-treated or differentiation cultures (not shown). For both c-Kit/CD117 and CD71, the reduction in non-myeloid markers was NAM-concentration dependent. In 5mM NAM, the reduction in expression was nearly as much as that seen under standard differentiation conditions in the presence of myeloid growth factors.

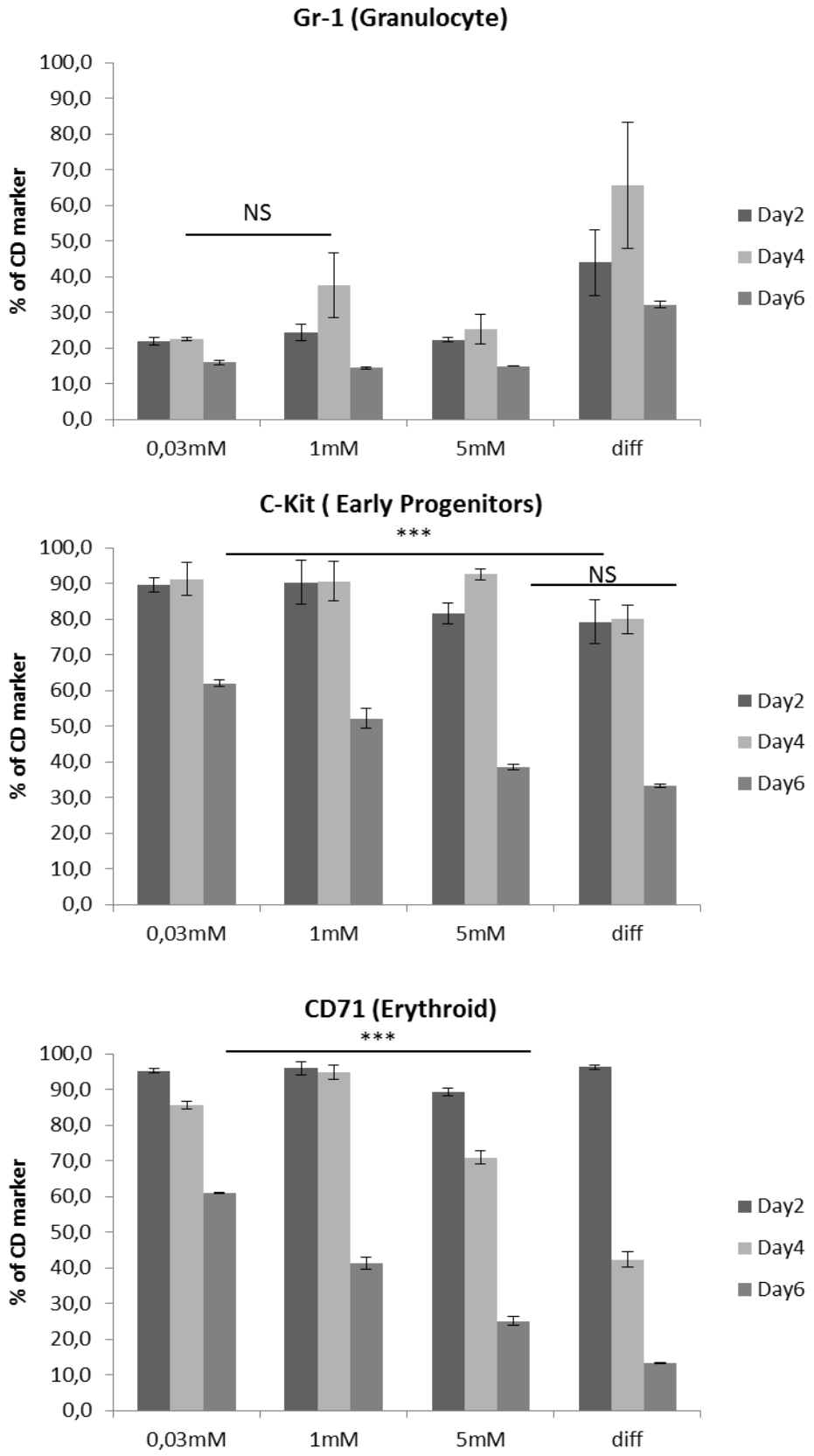


Fig 3-8E shows the effect of NAM on surface markers. Gr1 expression in the cultures supplemented with 1mM was only slightly (and not significantly) above the self-renewal control at day4 NAM reduce both C-Kit and CD71 during the later stages of culture: (% of CD markers : the proportion of events above a cut off (gate isotype control) n=3 ;NS, Not significant; ***, p = 0.000. Data expressed as the mean± SD).

3.9 NAM reduce the colonies formation and pSTAT5 signaling

The concentration-dependent loss of c-Kit under NAM treatment of FDCP-Mix suggests that NAM exposure may be causing a loss of stem/progenitor characteristics. To look at functional consequences of NAM-exposure in more detail, both the colony-forming potential and the JAK-STAT response to IL-3 were determined in FDCP-Mix cells pretreated with NAM for 4 days (Fig 3-8F)

NAM treatment reduced the colony-forming/self-renewal potential of FGDCP-Mix in a concentration-dependent manner, comparable with the loss of c-Kit expression, confirming that NAM causes loss of stem/early progenitor cell function as well as marker expression.

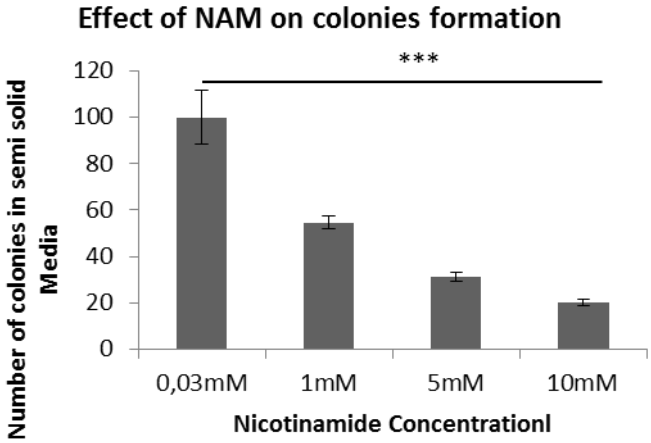


Fig 3-8F NAM treatment reduced the colony-forming / self-renewal potential of FGDCP-Mix. (n=3 ;p = 0.000. Data expressed as the mean± SD).

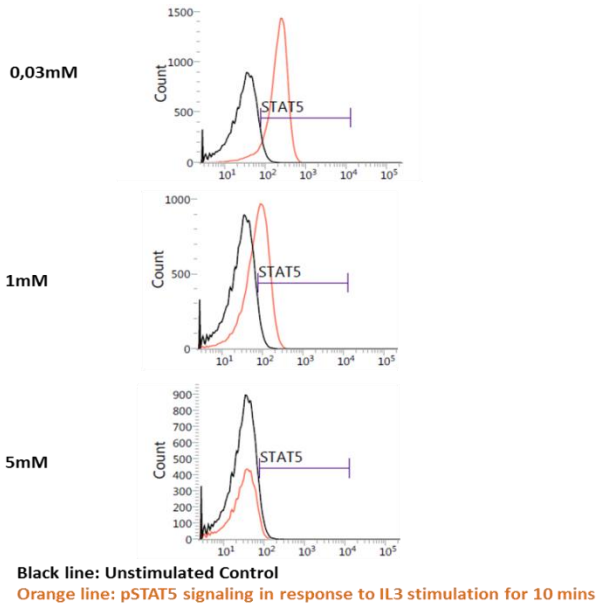


Fig 3-8G. Reduction of pSTAT5 signaling in NAM treated cells. The figure shows a representative result from 3 independent experiments.

Similarly, phosflow analysis of the pSTAT5 response of NAM-treated and control cells to IL-3 clearly showed the pSTAT5 signal to be reduced in NAM concentrations of 1mM or above (Fig 3-8G)

Taken together, these results show that 1mM NAM reduces the pSTAT5 signaling response to IL-3, cell cycling and self-renewal potential while initiating an atypical program of myeloid differentiation that results in granulocyte-like cells with abnormally low GR1 expression and apoptosis. Higher concentrations promote a more marked loss of stem/progenitor characteristics followed by cell death, rather than myeloid differentiation.

4. Discussion

The fate of HSC and progenitor cells is regulated through both extracellular and intracellular factors. The bone marrow niche environment provides specific cellular interactions with matrix, including physico-chemical and metabolic environments (Boulais and Frenette 2015; Ng and Alexander 2017; Pinho and Frenette 2019). The aims of this study were firstly to determine whether cellular metabolism affects the balance between signaling pathways affecting self-renewal and differentiation; secondly, to examine the dependence of signaling on changes in osmolarity and finally to determine the effects of nicotinamide on the self-renewal and differentiation of haematopoietic progenitor cells.

Previous publications have reached conflicting conclusions concerning the influence of nicotinamide (NAM) on HSCs/MPPs. On the one hand, a high NAM concentration has been shown to enrich for stem cells in cord blood-derived mononuclear cell preparations (Peled et al. 2012). On the other hand, NAM used at a somewhat lower concentration supports granulocyte differentiation in vitro and in vivo (Skokowa et al. 2009) . The study reported here found no evidence that NAM supports undifferentiated stem cells. Rather, the results reported here confirm and extend the work of (Skokowa et al. 2009) by showing that NAM drives a pattern of granulocyte differentiation that takes place even in the absence of added myeloid growth factors. It is possible that this is a result of induction of an autocrine loop of G-CSF and G-CSFR via NAD-dependent activation of sirtuin as suggested by (Skokowa et al. 2009). In this case, the cell cycle block (which was also noted by (Wang et al. 2018)) may be part of the differentiation program. Alternatively, the cell cycle block and granulocyte differentiation may be independent effects, as suggested by the stronger block without more marked differentiation at higher NAM concentrations. This finding suggests that the selective enrichment of stem cells in cord blood mononuclear cell preparations that was reported by (Peled et al. 2012) may in fact result from a combination of cell cycle block and general cytotoxicity to which more differentiated cells are more susceptible. Further work will be necessary to distinguish these effects and to characterize the functionality of the Gr1-ve granulocytes arising from NAM-induced differentiation in the absence of G-CSF. It will be particularly interesting to see whether these cells also arise from primary human progenitors treated with NAM in the absence of G-CSF.

The finding that salt supplementation of media increases the efficiency of FDCP-Mix colony formation in semi-solid media provides further evidence that balance between self-renewal and differentiation is not either entirely intrinsic or growth factor dependent, but can be influenced by other environmental factors. There has been very little published concerning the effects of osmolarity or salt concentration on HSCs in-vivo or in-vitro. Although increased osmolarity (adjusted with NaCl) has been noted to be advantageous for the maintenance of stem cells in long term culture (Coutinho et al 1993), it is not clear why a relatively small change in the salt concentration should have such a marked effect on the cells. A possible explanation for this phenomena may be the influence of the high salt concentration on amino acids transporter activity (Salazar et al. 2016). High osmolarity is known to increase

amino acid uptake, which may in turn affect the cell metabolism and somehow favor the self-renewal capacity (Yamazaki and Nakauchi 2015). Alternatively, the observed effects may be due to high salt being a particular disadvantage for maturing cells, that may be more sensitive to osmotic stresses (Ting-Beall et al. 1993).

It is particularly interesting that an increase in osmolarity caused either by salt or by mannitol increases the pSTAT5 response to IL-3. Since STAT5 signaling is associated with self-renewal rather than differentiation, this may provide an explanation for the increased colony formation in high salt media. In this case, however, it is unclear why colony formation was not increased in high mannitol medium. It is possible that this is due to a different duration of the increased signaling following the change in osmolarity and it would be interesting to compare the duration of the increased pSTAT5 response under high salt and high mannitol to distinguish between short term and long term responses.

Importantly, it is shown here that the balance of signals transduced from the IL-3 receptor in haematopoietic progenitor cells can be influenced by the metabolic environment and the energetic state of the cell. Reducing glucose reduces the Ras-MAPK response to IL-3 but actually increases the JAK2-pSTAT5 response. The high pSTAT5 signal under low intracellular ATP may in theory be either a part of programed cell death or autophagy (Majri et al. 2018; Altman and Rathmell 2012; Elmore 2007). However, due to the short time of incubation with metabolic inhibitors or low glucose before the Phosflow analysis (Fig3.1-3.3), this is unlikely to be the case here. It is more likely that a short term change in the pattern of metabolism shifts the relative activities of the JAK-STAT and Ras-MAPK pathways, so that self-renewal JAK-STAT signaling is favored at low energy levels. Interestingly, other signaling pathways associated with the maintenance and self-renewal of stem cells such as those of Wnt, Notch, Hedgehog and TGFß (Warr et al. 2011) also tend to be short and direct, and most probably require less energy than do the multi-stage kinase cascades such as the Ras-MAPK and PI3K-mTOR pathways that drive proliferation and differentiation.

It should be noted that a low energy metabolic stem cell niche might not just support the self-renewal rather than the differentiation of stem cells, but could also contain their potentially tumorigenic self-renewal potential. Firstly, if active stem cells within a low-energy niche are ready to differentiate but are prevented from doing so by limiting energy, then any cell leaving the niche would immediately activate differentiation pathways and differentiate. Secondly, a niche in which the rate of stem cell self-renewal is metabolically restricted avoids selection for cells with mutations in receptors, signal transducers, transcription factors or cell cycle control machinery that would otherwise have given a selective advantage to a mutated stem cell clone. In this way, a metabolic niche could guard against the emergence of abnormal clones (Cross et al. 2008).

The observed towards JAK-STAT signaling at low energy levels poses the question of how this is achieved. The simplest explanation would be that the maintenance of JAK2-STAT5 signaling requires less ATP than does the maintenance of a Ras-MAPK response (see Fig4.1-

Hypothesis). However, the situation appears not to be this simple, since the activities of the two pathways did not appear to be dependent on the same pool of free ATP, Ras-MAPK being dependent on glycolysis while JAK-STAT5 signaling was more dependent on mitochondrial metabolism. The direct measurement of the ATP pool confirmed that JAK-STAT signaling was more dependent on mitochondrial function than on ATP concentration, indicating a qualitative difference in the way that these pathways are fueled.

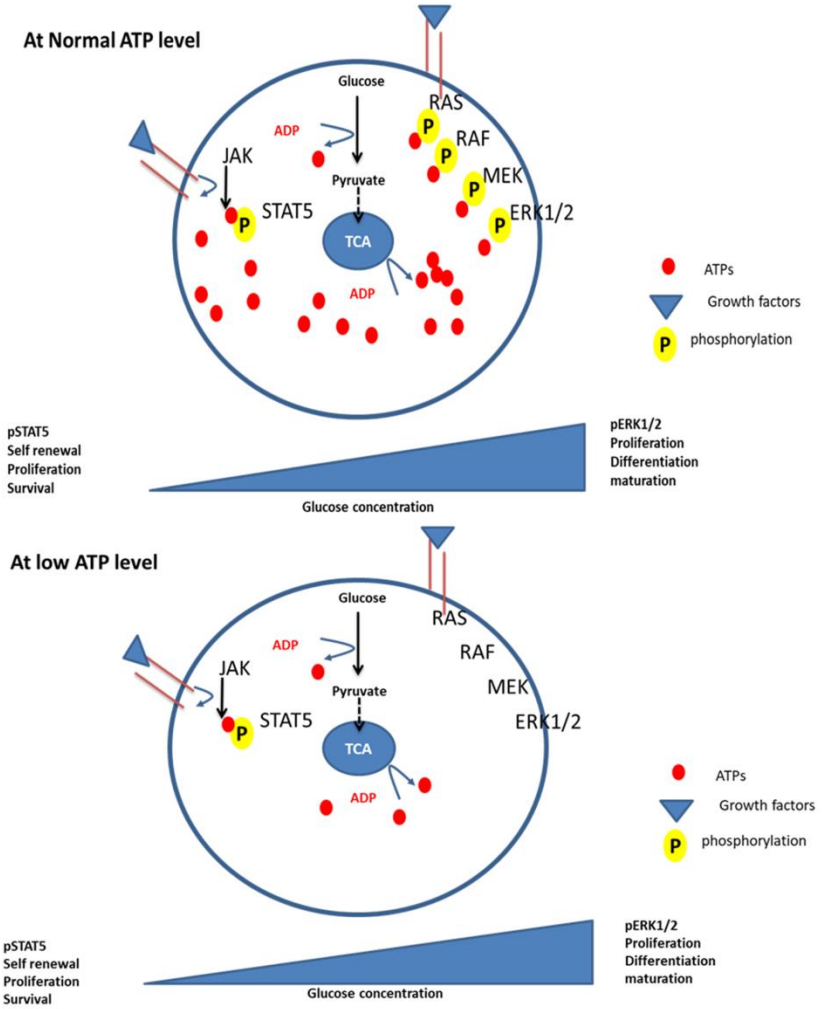


Fig4.1 The JAK- STAT5 pathway requires less ATP than the RAS-MAPK pathway. The upper panel shows the situation at high energy levels. Both the JAK-STAT and Ras-MAPK pathways have sufficient ATP and activity is dependent on the level of IL-3 bound receptor. The lower panel shows the siutaion at low energy levels.

It therefore seems likely that the JAK-STAT pathway can be maintained by a specific reaction that is not available to the Ras-MAPK pathway. The most obvious candidate reaction is the local regeneration of ATP from ADP and an NTP by a nucleoside diphosphate kinase (NDP Kinase). The local recharging of NTPs via NDP Kinases has been suggested to maintain enzyme activity in similar situations, and although the emphasis to date has been on regenerating GTP using ATP the simple reversibility of the NDPK reaction makes the regeneration of ATP using some other NTP equally feasible (see Fig 4.2) (Stahl et al. 1991; Schaertl et al. 1998; Vlatković et al. 2015). Since GTP is generated by mitochondrial

metabolism but not by glycolysis, this may explain why JAK-STAT signaling in low energy FDCP-Mix is more dependent on mitochondrial than on glycolytic metabolism.

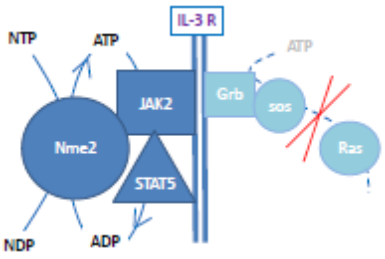


Fig4.2 The energy restriction supports selective STAT5 phosphorylation via local ATP regeneration by NDPKB.

Nucleoside diphosphate kinase B also known as Nme2/Nm23H2, Nm23B, NDKB and PUF is an enzyme that in humans is coded by the nme2 gene. Nme1 (NDPKA) and Nme2 (NDPKB) show 88% amino acid sequence similarity and are located in the same region of chromosome 17q21 (Stahl et al. 1991). The Nme2 protein has a nucleotide-binding site, kinase activity and also a DNA binding site which is independent from the other domains (Dexheimer et al. 2009). One of the main functions of Nme2 is to transfer the gamma phosphate group from ATP to non-adenosine NDPs to generate NTP or vice versa. Furthermore, Nme2 also acts as a transcriptional activator of c-myc (Postel et al. 1993). Importantly, both Nme1 and Nme2 have been described as differentiation inhibitory factors in AML and CML (Okabe-Kado et al. 1998; Yokoyama et al. 1996).

The knockdown of Nme2 in FDCP-Mix cells by transfection with a lentiviral shRNA expression vector confirmed that Nme2 is required to maintain a high level of JAK2-STAT5 signaling under low energy conditions. This demonstrates the existence of a refined NDPK-dependent mechanism allowing specific kinases to draw on energy reserves that are not available to other pathways. Knocking down the Nme2 reduced the pSTAT5 signaling only when the cells were under metabolic stress. In this regard, it is interesting that glucose restriction resulted in both an increase in Nme2 mRNA level in vector-control cells and a more marked reduction in Nme2 shRNA-expressing cells, suggesting that there may be an induction of Nme2 expression as well as increased turnover under energy-limiting conditions. The regulation of the Nme2 gene does indeed seem to be complex: a marked reduction in mRNA following transient transfection with shRNA expression plasmid leads to a rapid compensatory upregulation of the mRNA (Enrica Bach and Michael Cross, personal communication). Also, previous studies by (Tschiedel et al. 2012) found the expression of the fusion oncoprotein BCR-ABL in chronic myeloid leukemia to be accompanied by an extreme accumulation of Nme2 protein in the cytoplasm even though mRNA was barely detectable under these conditions, while the situation in AML is quite the opposite with high levels of Nme2 mRNA in the absence of detectable protein (Bach et al. 2012). These observations, together with the presence of Nme2 binding sites in the Nme2 gene promoter region, (Postel et al. 1993; Okabe-Kado et al. 1998; Yokoyama et al. 1996) suggest that Nme2 expression is most probably subject to very tight regulation involving negative feedback.

While it is possible that Nme2 plays a role in stem cell maintenance under non-laboratory conditions, there is no obvious stem cell or myeloid defect in Nme2 knockout mice under laboratory conditions. However, there was a marked defect in erythropoiesis in the Nme2 knockout mice (Postel et al. 2009) that was confirmed using FDCP-Mix cells in the current study. This suggests that Nme2-mediated focusing of energy resources may be involved in maintaining the viability and/or the undifferentiated state of erythroid progenitors, perhaps by interacting with signaling from the EPO receptor which, like the IL-3 receptor, also signals through JAK2-STAT5 (Joosten et al. 2004).

Given the reported association between Nme2 expression and the type and prognosis of myeloid leukemia and the obvious interest in the underlying mechanisms, it was encouraging to find that a proportion of normal human CD34+ progenitors show an ability to maintain JAK-STAT5 signalling under low-energy conditions similar to the murine FDCP-Mix cells. It will particularly important in the future to determine the relevance of Nme2 expression to the ability of normal and leukemic progenitors to survive and self-renew at low energy levels, as this may offer important new opportunities for both diagnosis and therapy.

5. Summary

Dissertation zur Erlangung des akademischen Grades Dr. rer. med.

Titel :

Metabolic Environment and Cellular Signaling in Haematopoietic Stem Cells and Progenitor Cells

eingereicht von

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Leipzig, Sep 2019

The adult human blood system is maintained by replacement of more than 10^{11} cells per day through continuous haematopoiesis: A hierarchical differentiation and proliferation system in the bone marrow. At the top of the hierarchy are haematopoietic stem cells (HSCs) that cycle slowly to generate on average one replacement HSC and one multipotent progenitor. The multipotent progenitors then proliferate more rapidly, generating daughter cells that follow programs of commitment to the lymphoid, myeloid or erythroid lineages. Under some conditions (during development or after damage), the pool of HSCs can be increased by symmetric divisions in which one stem cell produces two (Nakamura-Ishizu et al. 2014; Ng and Alexander 2017; Pinho and Frenette 2019).

It is important to understand the mechanisms regulating the production of blood cells (Haematopoiesis) in the bone marrow, in order to improve the diagnosis of haematological disease and to identify new opportunities for therapy.

The major focus to date has been firstly on the genes that control survival, differentiation and proliferation and that are commonly mutated in haematological disease, and secondly on a range of growth factors that are provided in the haematopoietic cell environment and can affect these processes by activating specific receptors.

At the same time it has been realized that the haematopoietic bone marrow is an organized structure in which haematopoiesis is arranged in specific areas or niches, at various distances from bone surface, arteries or sinusoid veins (Naito et al. 1992; Morrison and

Scadden 2014; Kumar et al. 2018). This organization suggests that the metabolic environment, including the availability of nutrients and oxygen as well as variation in osmolarity and pH, may vary with position and may therefore play a role in organizing and maintaining haematopoiesis.

Here, three aspects of the influence of the metabolic environment on the biology of haematopoietic progenitor cells have been investigated using a multipotent murine progenitor cell culture system (FDCP-Mix) that reproduces many features of normal progenitor cell self-renewal and differentiation.

Firstly, following up on conflicting reports that nicotinamide (NAM, a form of vitamin B3) can maintain self-renewing stem cells on the one hand but on the other hand increases granulocyte differentiation (Skokowa et al. 2009; Peled et al. 2012) , it was shown that NAM does not support self-renewing FDCP-Mix, but does support granulocyte differentiation. Surprisingly, the addition of NAM in the absence of added myeloid growth factors is sufficient to generate granulocyte-like cells. However, these granulocytes appear to lack the expression of the granulocyte-specific surface marker Gr1. This suggests that NAM can initiate a program of differentiation, but completion of the program requires an external source of G-CSF. It is unclear whether NAM concentrations change significantly in the bone marrow at different locations or at different times.

Secondly, the effects of osmolarity on self-renewal and differentiation were investigated, since osmolarity is likely to vary within haematopoietic marrow and has been previously reported to affect the maintenance and function of stem cells (Coutinho et al 1993). It was found that the positive effects of high osmolarity on the maintenance of multipotent progenitor FDCP-Mix are seen only when osmolarity is raised by the addition of salt (sodium chloride or potassium chloride) and not when raised by the addition of the inert sugar mannitol. This suggests that high salt, rather than osmolarity alone, favors self-renewing cells over differentiating cells. Potential mechanisms include the activation of amino acid transporters, effects on metabolism and/or signaling. Interestingly, high salt increased the level of JAK2-STAT5 signaling via the IL-3 receptor, which is associated with self-renewal rather than differentiation. However, a similar effect was seen in high mannitol, at least in the short term, meaning that a short term increase in the JAK-STAT response is not sufficient to favor self-renewing cells over differentiating cells in the FDCP-Mix system.

Thirdly, a more detailed analysis of the dependence of signaling activities on metabolism was carried out by comparing the JAK-STAT and Ras-MAPK (Erk1/2) response of FDCP-Mix to IL-3. Here, it was found that a simple decrease in the glucose concentration of the medium resulted in a shift towards JAK-STAT signaling. By using selective inhibitors of glycolysis and mitochondrial electron transport, it was further demonstrated that Ras-MAPK pathway activity varies with the concentration of free ATP and is largely dependent on glycolysis. In contrast, JAK-STAT pathway activity was maintained at low ATP levels and was mainly dependent on mitochondrial metabolism rather than on glycolysis. This suggested that the

two pathways do not rely equally on the same energy source. These observations led to the hypothesis that the JAK-STAT pathway may be maintained by the activity of a nucleoside diphosphate kinase that can regenerate ATP from ADP locally by transferring the gamma phosphate from GTP. In support of this hypothesis, a requirement for the NDP kinase Nme2 was demonstrated by knockdown of endogenous Nme2 in FDCP-Mix cells using a shRNA lentivirus construct. Reduction of the Nme2 level led to a reduction in the JAK-STAT signaling activity under low-energy conditions, while signaling under high energy conditions was not affected. This demonstrates a previously unidentified link between signaling and metabolism that is likely to be relevant for the maintenance of stem cell fate in low energy niches. The previously reported overexpression of Nme2 protein in chronic myeloid leukemia progenitor cells and the association of Nme2 mRNA expression with prognosis in acute myeloid leukemia underline the probable relevance of Nme2 to the maintenance of stem cells in low-energy niches in both normal and leukemic haematopoiesis.

This work shows how the nutrient and physico-chemical environment can indeed affect the self-renewal versus differentiation of haematopoietic progenitor cells in vitro, consistent with the proposed existence of metabolic niches in vivo that contribute to the organization and control of haematopoiesis. The identification of Nme2 as a key link between metabolic and signaling activities should enable more detailed analysis of these relationships in the future.

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Appendix I

Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

09.09.2019 

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Appendix II

Acknowledgment

I would like to express my sincere gratitude to Prof. Ulrich Sack and PD.Dr. Michael Cross whom I am indebted for their supervision, advices, their patience, motivation and immense help especially. Their support overwhelms me and no words can describe my appreciation for them. They simply changed me not just academically but also personally. Beside my advisors, I would like to thank Prof. Sunna Hauschildt , Prof. Uwe Platzbecker and Prof. Gerhard Behre for their insightful comments and hard questions which incented me to widen my research.

Of course my sincere thanks go to Nicole Noack , Dr. Enrica Bach, Michael Bitar, Dr. Alexander Wurm, Christian Böhme , Franziska Wike ,Johannes Küpper, Samuel Kindde Birru and Amanpreet Kaur Pains for their techniqual support and for stimulating discussion. Without their precious support it would not be possible to conduct this research. I am grateful for my teachers, colleagues and friends for their patience and valuable help. I also would like to extend my thanks to the staff of the haematology department and the flowcytometery core facility at University clinical Leipzig.

In closing, beside my colleagues at University of Khartoum I am very appreciated to the Sudanese Ministry of Higher Education and Researches. Without the scholarship which I hold, this project might not exist.

Last but not the least, I would like to thank my family: my mother , my sister ,my brothers , my wife and my little daughter for supporting me spiritually throughout writing this thesis and in my life in overall.